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Male Reproductive Health and Environmental Xenoestrogens

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Male reproductive health has deteriorated in many countries during the last few decades. In the 1990s, declining semen quality has been reported from Belgium, Denmark, France, and Great Britain. The incidence of testicular cancer has increased during the same time. Incidences of hypospadias and cryptorchidism also appear to be increasing. Similar reproductive problems occur in many wildlife species. There are marked geographic differences in the prevalence of male reproductive disorders. While the reasons for these differences are currently unknown, both clinical and laboratory research suggest that the adverse changes may be inter-related and have a common origin in fetal life or childhood. Exposure of the male fetus to supranormal levels of estrogens, such as diethlylstilbestrol, can result in the above-mentioned reproductive defects. The growing number of reports demonstrating that common environmental contaminants and natural factors possess estrogenic activity presents the working hypothesis that the adverse trends in male reproductive health may be, at least in part, associated with exposure to estrogenic or other hormonally active (e.g., antiandrogenic) environmental chemicals during fetal and childhood development. An extensive research program is needed to understand the extent of the problem, its underlying etiology, and the development of a strategy for prevention and intervention. — Environ Health Perspect 104(Suppl 4):741–803 (1996)

Key words: male reproduction, reproductive disorders, semen quality, testicular neoplasms, environment, estrogenic chemicals, endocrine disruptors, exposure, pesticides

Introduction

In 1992, a research group at the Department of Growth and Reproduction, the National University Hospital (Rigshospitalet), and the Panum Institute, Copenhagen, Denmark, published in the *British Medical Journal* a metaanalysis of the data from the international literature that revealed a significant decrease in sperm concentration and semen volume in otherwise normal men over the period 1938 to 1990. During the same time period, the incidence of testicular cancer had markedly increased in many countries. These and other observations provided a clue that this apparent decline in male reproductive health might be caused by some common environmental factors. It was recognized that similar abnormalities of the male reproductive system were caused by administration of estrogens during pregnancy in humans and experimental animals; therefore, a hypothesis was put forward that environmental chemicals having estrogenic effects were contributing agents. In particular, it was suggested that fetal exposure to an excess of estrogenic compounds was a key risk factor.

After an intense public debate in the Danish news media on the possible role of environmental chemicals, such as pesticides, detergents, plasticizers, and other industrial chemicals, the Danish Environmental Protection Agency (DEPA) of the Ministry of Environment and Energy in September 1994 decided to support the preparation of a review summarizing the current knowledge on male reproductive disorders and environmental chemicals with estrogenic effects. In addition, the review was to identify gaps in knowledge and address research needs and requirements in order for researchers to perform adequate risk assessments. The DEPA asked Professor Niels E. Skakkebæk, Department of Growth and Reproduction at the National University Hospital and John Chr. Larsen, Division Head, Institute of Toxicology at the Danish National Food Agency to prepare the report. The project received additional

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Abbreviations used: ADI, acceptable daily intake; AFP, α–fetoprotein; Ah, aryl hydrocarbon; APE, alkylphenol polyethoxylate; BBP, butylbenzyl phthalate; BHA, butylated hydroxyanisole; BHC, benzene hexachloride; bw, body weight; CIS, carcinoma *in situ*; CNS, central nervous system; CP, cyclophosphamide; 2,4-D, 2,4-dichlorophenoxyacetic acid; DBCB, dibromochloropropane; DBP, di-*n*-butyl phthalate; DDD, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane; DDE, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene; DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; DEHP, di(2-ethylhexyl)phthalate; DEPA, Danish Environmental Protection Agency; DES, diethylstilbestrol; EBDC, ethylenebisdithiocarbamate; EHC, Environmental Health Criteria; EU, European Union; FDA, U.S. Food and Drug Administration; FSH, follicle-stimulating hormone; GAP, Good Agricultural Practice; GLP, Good Laboratory Practice; HCB, hexachlorobenzene; HCH, hexachlorocyclohexane; *(continued)*

financial support from the Ministry of Health through its National Research Centre for Environmental Medicine.

Dr. Jorma Toppari, Departments of Pediatrics and Physiology, University of Turku, Finland, prepared a draft of the report. This draft was then discussed by a group of Danish and international experts invited to a one-week workshop that was held at Rigshospitalet in Copenhagen, January 23–27, 1995. The participants who are the authors of the present publication actively contributed to the endeavor both during the workshop and afterward. The final manuscript was edited by Jorma Toppari together with Niels E. Skakkebæk and John Chr. Larsen. The present review is a revised version of the official report (*1*), which was printed by the Danish Ministry of Environment and Energy mainly for circulation in Denmark.

The review addresses the possible effects of environmental chemicals known to possess estrogenic activity on male reproductive health. The term xenoestrogen is often used for such compounds, whereas the term synthetic estrogens refers to medical drugs mainly used for contraception and treatment of various diseases.

A number of other environmental chemicals have been implicated as environmental hormones or endocrine disruptors. Although not shown (and in many instances not adequately tested) to possess direct estrogenic activity, some of these compounds may in some cases also affect male reproduction. The mechanisms of action are not known in detail but they may involve, for example, antiandrogenic activity; modulatory effects on enzymes controlling sex hormone metabolism; or direct influence on the hormone-producing organs such as the thyroid gland, pituitary gland, and adrenal glands. These compounds may also affect estrogen levels through indirect feedback mechanisms.

The authors are well aware that the decline in semen quality and the increase in the incidence of testicular cancer may be caused by many other environmental, life-style, or genetic factors. For example, some chemicals that are now known as occupational toxicants were shown to affect the semen quality of the workers through a toxic action on the gonads, without any apparent estrogenic effects. Such toxic effects are not the object of the present report, but should be kept in mind in any consideration or scientific investigation of the adverse effects of environmental chemicals on male reproductive health.

Secular Trends in the Incidence of Male Reproductive Disorders

Trends in Semen Quality

Several reports in the literature have suggested a possible decline in human semen quality during the last 50 to 60 years (*2–4*). However, most of these reports were based on data from men attending infertility clinics or from very selected groups of fertile men and, therefore, the decline in sperm counts was presumed just to reflect changes in the policy of infertility treatment or a bias in selection of patients rather than a true biological phenomenon. A systematic metaanalysis of 61 studies that included 14,947 normal men revealed a significant decrease in sperm concentration (113 million/ml vs 66 million/ml; Figure 1) and semen volume (3.40 ml vs 2.75 ml) over the period of 1938 to 1990 (*5*). This report stimulated extensive discussion and some criticisms on the basis of possible technical errors and known limitations of metaanalysis (*6,7*). Carlsen and co-workers responded to these criticisms (*8*). Although the data for 1970 to 1990 were compatible with a decrease as well as with no change or an increase in semen quality, the cautious general conclusion was that a real decline occurred during the 50-year period (*9*). The findings of Carlsen et al. (*5*) were also compared (*6*) to those of MacLeod and Wang (*10*) from the United States. This comparison is not relevant, however, because the metaanalysis was based on semen analyses of normal men, whereas the American study examined men who were clients of an infertility clinic.

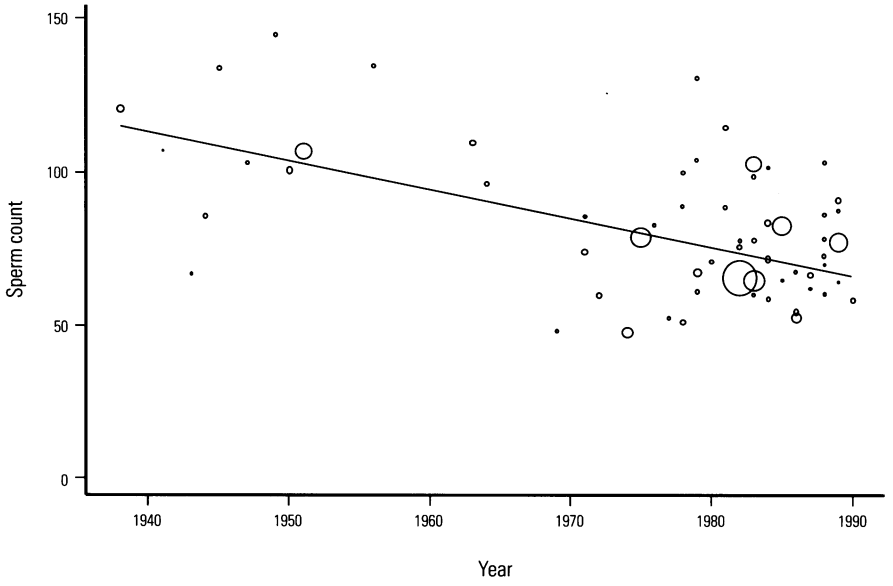


Figure 1. Linear regression of mean sperm density reported in 61 publications (represented by circles the area of which is proportional to the logarithm of the number of subjects in the study), each weighted according to number of subjects, 1938–1990. The figure is based on the data reported by Carlsen et al. (*5*). A corresponding figure in that paper was incomplete.

Abbreviations used *(continued)*: HPLC, high-performance liquid chromatography; JMPR, Joint FAO/WHO Expert Meeting on Pesticide Residues; LH, luteinizing hormone; LOAEL, lowest observed adverse effects level; MIS, Müllerian inhibiting substance; MRL, maximal residue limits; NOAEL, no observed adverse effect level; NOEL, no observed effect level; NP, nonylphenol; OECD, Organisation for Economic Cooperation and Development; OP, octylphenol; OPPT, Office of Pollution Prevention and Toxics; OPPTS, Office of Prevention, Pesticides and Toxic Substances; PCB, polychlorinated biphenyl; PCDD, polychlorinated dibenzo-*p*-dioxin; PCDF, polychlorinated dibenzofuran; PCP, pentachlorophenol; PCT, porphyria cutanea tarda; PE, proliferative efficiency; PNS, peripheral nervous system; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; RfD, reference dose; RPE, relative proliferative efficiency; RPP, relative proliferative potency; SHBG, sex hormone-binding globulin; STW, sewage treatment water; TBT, tributyltin; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD-TEQ, TCDD equivalency; TDI, tolerable daily intake; TEF, toxic equivalency factor; TWI, tolerable weekly intake; U.S. EPA, U.S. Environmental Protection Agency.

The metaanalysis of Carlsen et al. (*5*) prompted several laboratories to evaluate their data on the quality of semen obtained during recent years. In a French study of 1351 healthy men volunteering for sperm donation, a 2.1% decrease in sperm concentration per year from 89 million/ml in 1973 to 60 million/ml in 1992 ($p<0.001$) was found (*11*). Furthermore, the percentages of motile and normal spermatozoa also decreased significantly (Figure 2), whereas semen volume remained unchanged (3.8 ml). It is notable that the year of birth of the study subjects contributed significantly to the results. Multiple regression analysis (which allows for separate effects of age and calendar year at birth) showed yearly decreases of 2.6% in sperm concentration, 0.3% in motility percentage, and 0.7% in the percentage of normal spermatozoa according to the year of birth of the men (all changes, $p<0.001$) (*11*). Similar results were obtained in a Scottish study (*12*) of 577 semen donors where a correlation was found between the median sperm count and the year of birth; the median sperm concentration decreased from 98 million/ml among donors born before 1959 to 78 million/ml among donors born after 1970 ($p=0.002$). The total number of sperm in the ejaculate fell from 301 million to 214 million ($p=0.0005$)(*12*). The association between declining semen quality and a more recent year of birth lends support to the concept that adverse prenatal factors may influence the sperm production capacity in adult life. Deterioration of sperm counts as well as motility among semen donor candidates during the past two decades was also observed in a smaller Belgian study (*13*). Ginsburg and Hardiman (*14*) reported a decrease in sperm concentrations (105 million/ml in 1978–1983 vs 76 million/ml in 1984–1989) of the partners of women treated for infertility and living in the Thames water supply area of London, whereas no decrease was found among those who lived in other water supply areas of London. However, the mean percentage of abnormal spermatozoa increased in all areas during the study period (18–19% vs 30–32%) (*14*). The data in all the studies cited above originated in individual laboratories that used consistently the same methods for semen analysis throughout the period.

The decreasing trend in semen quality may not be global. In contrast to the Paris area, no change in sperm concentration was found in the Toulouse area in France during 1977 to 1992 (*15*). The mean sperm count of samples from 302 healthy fertile donors was 83 million/ml (*15*). Furthermore, the sperm concentration in semen of Finnish men has remained unchanged between 1958 and 1992 (111 million/ml vs 124 million/ml) and is higher than elsewhere in Europe (*16*). It is of interest that the incidence of testicular cancer, and perhaps also hypospadias, in Finland is much lower than that in other Nordic countries (below), suggesting that these phenomena may be related in some way. The reason remains unknown, but further examination may provide important clues to the etiology of decreasing sperm quality worldwide. Urban areas (e.g., Paris) appear to have a declining trend in sperm counts, whereas rural areas (e.g., Toulouse or Finland) seem to have stable sperm concentrations in semen.

Incidence of Testicular Cancer

Testicular cancer is now the most common malignancy of young men in many countries; and although it is still rare compared to the malignant diseases most prevalent in old age, the lifetime risk of developing testicular cancer now approaches 1% in a country such as Denmark. The incidence of testicular cancer has increased for several decades (*17*). On the basis of data from cancer registries, increases in incidence are evident in England and Wales (*18,19*), Scotland (*20*), the Nordic and Baltic countries (*21,22*), Australia (*23*), New Zealand (*24,25*), and the United States (*26*). The observed increase has been approximately 2 to 4% per annum in men under 50 years of age (Figures 3, 4) and occurred in the same age group in which testicular cancer incidence peaks, i.e., young adults (Figure 5). Table 1 displays the changes that have occurred during the last 25 years. There are marked racial and geographic differences. For example, Denmark has a 4-fold higher incidence of testicular cancer than does nearby Finland, and Caucasians are 3-fold more susceptible to this disease than are African Americans in the United States. Nevertheless, it is obvious that there is a worldwide trend toward an increased incidence of testicular cancer as illustrated in Figures 3–5. The incidences of both seminomas and nonseminomas have increased (*17*). Mortality due to testicular cancer increased from the beginning of this century until the early 1970s when, because of the development of good medical treatment, mortality began to decline (*17*). However, we still do not know the etiology of testicular cancer and cannot therefore develop any preventive measures.

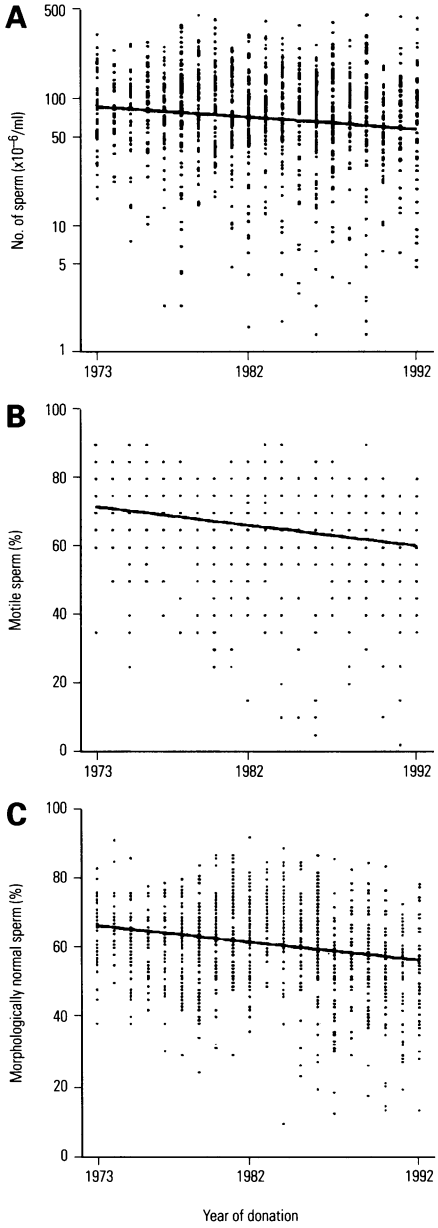


Figure 2. Changes in the sperm concentration (*A*),the percentage of motile sperm (*B*), and the percentage of morphologically normal sperm (*C*) in 1351 fertile men, 1973–1992. Linear regression analysis revealed a decrease of 2.1% per year in the mean sperm concentration, from 89×10^6 per milliliter in 1973 to 60×10^6 per milliliter in 1992. The concomitant decreases in the mean percentages of motile and normal spermatozoa were 0.6 and 0.5% per year, respectively. Reproduced with permission from Auger et al. (*11*).

Incidence of Cryptorchidism

Birth data from several reports have indicated a substantial increase in the incidence

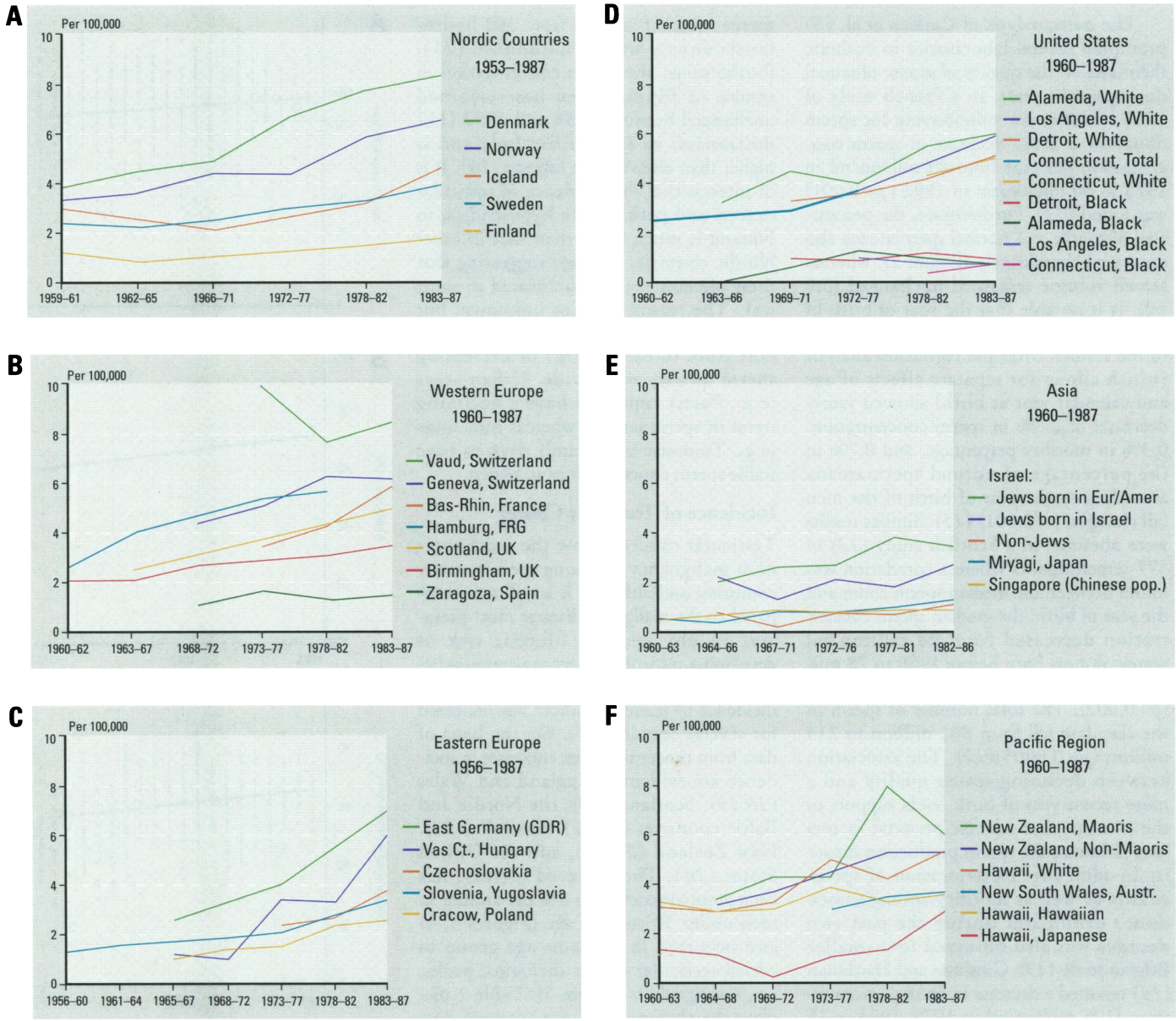


Figure 3. Secular, racial, and geographic trends in the incidence of testicular cancer, 1953–1987. Compilation of data from IARC (*318–323*).

of cryptorchidism (maldescent of the testis). However, estimates of the prevalence of cryptorchidism obtained from different studies are difficult to compare. It is often not clear how a cryptorchid testis was defined, and inclusion of different proportions of boys with retractile testes could account for the reported differences. The sources of data used in these reports also differ considerably. The prevalence rates have varied between 0.03 and 13.4% on the basis of data from birth to 1 year of age from hospital or central registers (including different proportions of preterm babies) (*27–48*); 0.16 to 13.3% in surveys from school, army, etc. (*36,38,49–57*); and 2 to 4.7% in cohort studies based on discharge diagnosis (*41,58,59*). A few studies include ethnic data on non-Caucasians: birth data from India (*33*), Formosa (Taiwan) (*38*), and Korea (*44*) indicated prevalences of 1.6, 1.4, and 0.7% of cryptorchidism, respectively. A school survey from Nigeria (*56*) indicated a prevalence of 0.5%. The incidence of cryptorchidism among African Americans was reported to be only one-third that among whites (*34*), although another study (*48*) did not find a significant difference. Racial and ethnic data are pooled in most studies. Unfortunately, very few studies exist examining temporal changes in the incidence of cryptorchidism, confined to the same population and geographic areas and using an identical definition of the condition.

Discharge data from the Hospital Inpatient Enquiry from England and Wales showed that the proportion of boys undergoing orchidopexy (operation to bring the testis into the scrotum) before 15 years of age increased from 1.4% for a 1952 birth cohort to 2.9% for a 1977 birth

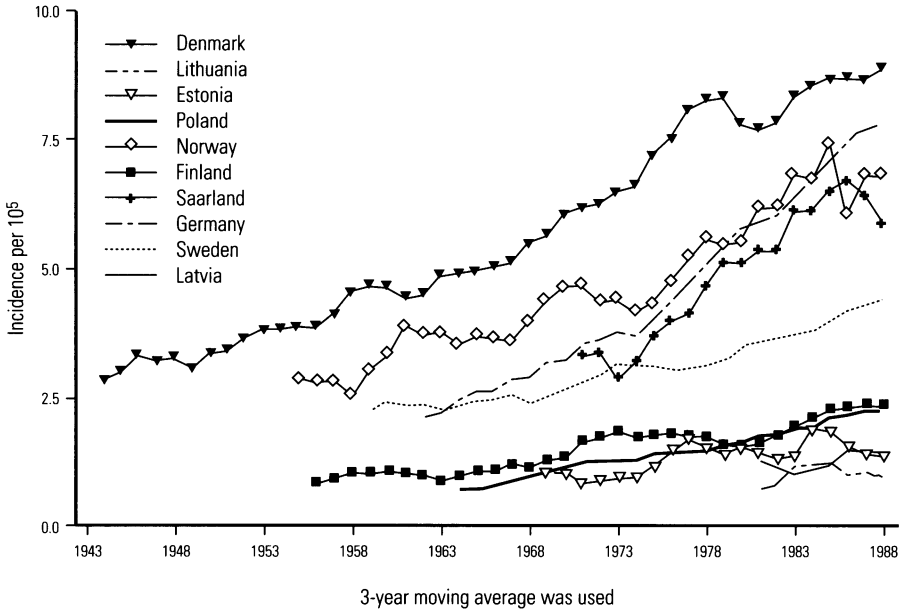


Figure 4. Trends in age-standardized (world standard population) incidence rates of testicular cancer. From Adami et al. (*22*); reprinted with permission from Wiley-Liss, Inc.

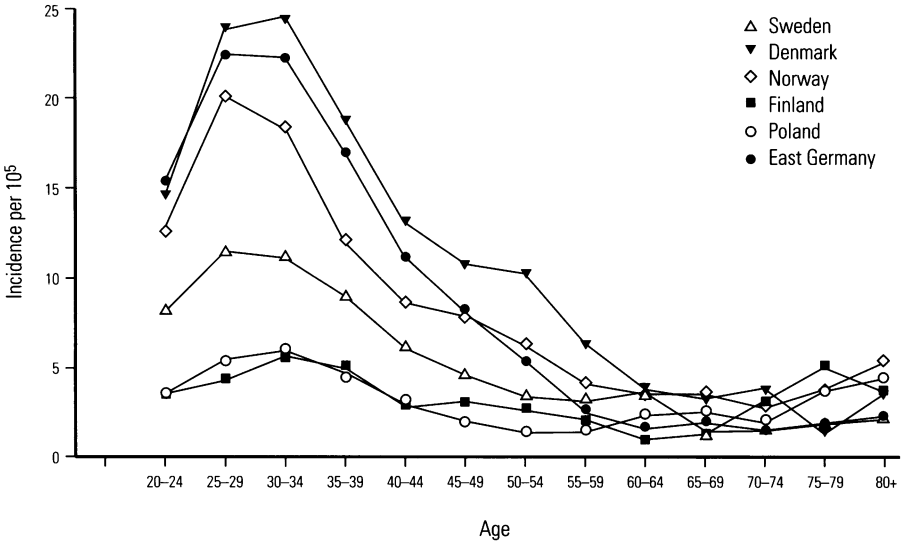


Figure 5. Age-specific incidence of testicular cancer 1985–1989 in the Nordic countries, Poland, and former East Germany. From Adami et al. (*22*); reprinted with permission from Wiley-Liss, Inc.

cohort (*58*). However, it is not known whether this is a reflection of a true increase in the prevalence of cryptorchidism or whether a considerable number of boys with retractile testes were undergoing orchidopexy. In Scotland, the annual number of discharges of boys 0 to 14 years of age with the diagnosis of cryptorchidism also showed a substantial increase during 1961 to 1985 (*41*). In Denmark, the prevalence of cryptorchidism at birth in male infants weighing > 2500 g varied between 1 and 1.8% in three different data sets obtained in the late 1950s (*60*). School surveys suggested higher prevalence rates up to 7% during 1940 to 1966, but these figures apparently included retractile testes (*55*). Cohort analysis of data from the Danish National Register of Hospital In- and Outpatients, from the period 1982 to 1985, indicated an incidence of cryptorchidism of approximately 2% (*59*).

In a study from the late 1950s that examined more than 3500 male infants delivered in a hospital in London and followed up to 1 year of age, Scorer (*31*) found that the incidences of cryptorchidism at 3 months of age in boys with birth weights < 2500 g and > 2500 g were 1.74 and 0.91%, respectively. Scorer used very accurate definitions of the positions of testes, and therefore, this well-conducted study has served as a reference for later research. In a large study from the 1980s, comprising 7441 male infants from Oxford (*47*), the very same examination technique and definitions of cryptorchidism were used; the rates of cryptorchidism at the age of 3 months in boys with birth weights < 2500 g and > 2500 g were 5.2 and 1.61%, respectively, indicating a significant increase compared to Scorer's figures. In another study from the late 1980s composed of 6935 male infants from New York (*48*) (using identical study techniques and case definition), prevalence rates of cryptorchidism at the age of 3 months in boys with birth weights < 2500 g and > 2500 g were 1.94 and 0.91%, respectively. However, the study population was racially and ethnically heterogeneous. From these three large studies, one can conclude that there has been a significant increase in the incidence of cryptorchidism in England, but the incidence in the racially and ethnically mixed population of New York is similar to that reported in the 1950s in England.

The epidemiological data indicative of an increasing incidence of cryptorchidism are not unequivocal. This important issue necessitates large regional prospective studies in which standard criteria are adopted for an accurate description of cryptorchidism.

Incidence of Hypospadias

Birth data from several reports have indicated a substantial increase in prevalence of hypospadias (Figures 6, 7). Figures of birth prevalence of hypospadias in the world literature vary considerably—from 0.37 to 41 per 10,000 infants (*61,62*)—and are difficult to compare. There are several factors that may contribute to the reported differences: different levels of ascertainment, different inclusion of minor forms of hypospadias or differences in ethnical origin of the population. As reported for cryptorchidism, very few longitudinal studies confined to the same population

Table 1. The incidence of testicular cancer, 1953 to 1987.[a]

	1960–1963	1964–1968	1969–1972	1973–1977	1978–1982	1983–1987
Nordic countries						
Denmark	3.8	4.5	4.9	6.7	7.8	8.4
Finland	1.2	0.8	1.1	1.7	1.5	1.8
Iceland	3	–	–	–	3.2	4.7
Norway	3.3	3.6	4.4	4.4	5.9	6.6
United Kingdom						
England and Wales						
Brimingham region	2.1	2.1	2.7	2.9	3.1	–
Birmingham and West Midlands	–	–	–	–	–	3.5
Liverpool region	2.1	2.2	2.4	–	–	–
Southern Metropolitan region	2.4	–	2.5	–	–	–
Southwest region	2.1	2.4	2.6	–	3.8	5
Northwest	–	–	–	3.2	3.3	4
Urban	–	–	–	3.3	–	–
Rural	–	–	–	2.9	–	–
Sheffield region	–	2.1	2.4	–	–	–
Oxford	–	2.5	2.6	3.7	3.9	4.4
South Thames	–	–	–	3.4	3.5	3.5
Trent	–	–	–	3	3.4	3.8
Mersey	–	–	–	2.3	4	–
Yorkshire	–	–	–	–	–	4.1
Scotland	–	2.5	–	–	4.4	5
East Scotland	–	–	–	3.6	3.8	5.3
North Scotland	–	–	–	4.3	4.2	4.4
Northeast Scotland	–	–	–	4	5.5	5.3
Southeast Scotland	–	–	–	4.3	5.3	5.1
West Scotland	–	–	–	2.9	3.9	4.8
Western Europe						
France						
Bas-Rhin	–	–	–	3.5	4.3	5.9
Urban	–	–	–	4.2	–	–
Rural	–	–	–	2.6	–	–
Doubs	–	–	–	2.3	3.4	3.8
Urban	–	–	–	2	3.3	–
Rural	–	–	–	–	3.8	–
Germany						
Saarland	–	–	3	3.4	5.4	6.2
Urban	–	–	–	–	5.5	–
Rural	–	–	–	–	5.4	–
Hamburg	2.6	4	4.7	5.4	5.7	–
Italy						
Varese	–	–	–	2.3	3.3	4
Parma	–	–	–	–	2.9	2.3
Ragusa	–	–	–	–	0.6	1.4
Netherlands						
3 provinces	2.8	–	–	–	–	–
Eindhoven	–	–	–	–	3.2	3
Spain						
Navarra	–	–	–	1.3	1.2	1.4
Zaragoza	–	–	1.1	1.7	1.3	1.5
Urban	–	–	–	1.6	–	–
Rural	–	–	–	2	–	–
Switzerland						
Basel	–	–	–	–	8.3	8.4
Geneva	–	–	4.4	5.1	6.3	6.2
Neuchatel	–	–	–	9.1	5.4	7
St. Gall	–	–	–	–	–	8
Vaud	–	–	–	9.9	7.7	8.5
Urban	–	–	–	8.9	9.9	–
Rural	–	–	–	10.5	6.5	–

(continued)

and geographic area exist. The increasing incidence of hypospadias has been reported primarily in England and Wales (*39*), Hungary (*63,64*), Sweden (*65–67*), Norway (*67,68*) and Denmark (*66,67*). No increasing trend was noticed in Finland, Spain, New Zealand, Australia, or Czechoslovakia (*67*).

England and Wales. The data from England and Wales are based on the national register that includes the whole population. Analysis of the data indicated a steady increase in the prevalence of hypospadias from 7.3 per 10,000 births in 1964 to approximately 16 per 10,000 births in the early 1980s, when the number of cases stabilized (Figure 6). In 1990 the prevalence of hypospadias showed a decrease to 11.7 per 10,000.

Hungary. The Hungarian data are based on the national register of the whole population. As shown in Figure 6, there was a rapid increase in the prevalence of hypospadias from 5.5 per 10,000 births in 1964 to 23.9 per 10,000 in 1978. Since then, the prevalence of hypospadias, although fluctuating, has remained at approximately the same level.

Scandinavian Countries. The data on the incidence of hypospadias in Scandinavia (Figure 7) are all based on the national registers that include the whole populations. The Danish data from 1970 to 1981 indicated a significant increase for this period (from approximately 7.5 to 12 per 10,000 births) (*66*). A further increase was noticed during the period 1982 to 1988 (*67*). However, this increase may be difficult to interpret as a new registration system was introduced. Nevertheless, this increase was similar to that reported for the years 1970 to 1981.

The data from Sweden also indicated a marked increase in the early 1970s: prevalence of hypospadias at birth was 40% higher between 1974 and 1982 than for the period 1965 to 1968 (*66,69*). However, the data obtained in the earlier period could be more complete, because they included both hospital records and registry data.

Also in Norway the prevalence of hypospadias at birth increased from 7 to 8 per 10,000 births between 1967 and 1971 to 13 per 10,000 in 1973 (*68*). In 1988, the prevalence was 20.7 per 10,000 births (*67*).

Ethnic Differences. The incidence of hypospadias in the United States is higher in Caucasians than in African Americans (ratio of 1.3–3.9:1) (*70–73*). In British Columbia, Canada, Native Americans were

Table 1. *(continued)*

	1960–1963	1964–1968	1969–1972	1973–1977	1978–1982	1983–1987
Zurich	–	–	–	–	7.4	8.8
Urban	–	–	–	1.9	–	–
Rural	–	–	–	1.4	–	–
Eastern Europe	–	–	–	–	–	–
Czechoslovakia	–	–	–	2.4	2.7	3.8
Urban	–	–	–	3.6	4.8	–
Rural	–	–	–	2.2	2.4	–
East Germany (GDR)	–	2.6	3.3	4	–	7
Hungary						
Szabolcs	–	1	1.8	1.5	1.2	2.7
Urban	–	–	–	–	1.7	–
Rural	–	–	–	–	1.1	–
Vas	–	1.2	1	3.4	3.3	6.1
Urban	–	4.1	4.6	4.7	6.6	–
Rural	–	3.3	4.3	4.2	5.5	–
Poland						
Cieszyn	–	–	–	2	–	–
Urban	–	–	–	2.8	–	–
Rural	–	–	–	1.5	–	–
Cracow city	–	1	1.4	1.5	2.4	2.6
Katowice	–	0.9	1.2	1.1	–	–
Urban	–	–	–	1.1	–	–
Rural	–	–	–	0.9	–	–
Nowy Sacz	–	–	–	1.1	1.2	1.2
Warsaw city	–	0.8	1.7	2.4	2	3.3
Warsaw rural	–	–	0.6	0.9	–	1.9
Romania						
County Cluj	–	–	–	1.3	1.3	1.4
Urban	–	–	–	–	1.7	–
Yugoslavia						
Slovenia	1.3	1.6	1.9	2.1	2.8	3.4
Canada	–	–	–	–	3	3.6
Alberta	3	3	3	3.8	3.6	3.7
British Columbia	–	–	3.1	3.3	3	4.2
Manitoba	3	2.7	3.1	3.4	4	4
Maritime Provinces	–	–	1.9	2.3	2.8	3
New Brunswick	1.6	1.4	–	–	2.7	2.6
Nova Scotia	–	–	–	–	3.2	3.2
Newfoundland	1.5	1.7	1.7	2.1	2.6	2.3
Ontario	–	–	–	2.7	3.4	4.2
Quebec	–	1.1	1.4	1.6	2	2.4
Northwest Territory and Yukon	–	–	–	5.5	–	4.8
Saskatchewan	2.2	3.3	3.5	3	3.3	4.5
United States						
SEER, White	–	–	–	–	–	4.9
SEER, Black	–	–	–	–	–	0.7
Connecticut	2.4	–	2.8	3.6	–	–
White	–	–	–	–	4.2	4.9
Black	–	–	–	–	0.2	0.6
California						
Alameda						
White	–	3.1	4.4	3.9	5.4	5.9
Black	–	0	0.5	1.2	0.9	0.7
Bay Area						
White	–	–	4.5	4	4.9	5.3
Black	–	–	1	1	1.1	0.8

(continued)

reported to have a lower incidence of hypospadias than the general Caucasian population, with a ratio of 1:6.7 (*74,75*).

Geographic Variation. Considerable variation exists in the prevalence of hypospadias among different countries. Interestingly, some populations with a low incidence of testicular cancer (e.g., Finland) (*22*) have a very low prevalence of hypospadias (Figure 7). Furthermore, there seems to be considerable geographic variation in the prevalence of hypospadias within different countries (*66,76*).

Incidence of Male Breast Cancer

As xenoestrogens were implicated as possible factors involved in the pathogenesis of breast cancer in women (*77*), the trends in the incidence of this disease in males should be monitored. Male breast cancer is a rare disease; only a few studies exist on geographical and temporal trends. Ewertz et al. (*78*) studied the incidence of male breast cancer in four Nordic countries and found a weak increase with calendar time in Denmark (1943–1982) but no change in Finland, Norway, and Sweden over the period 1953 (1958 for Sweden) to 1982. Ewertz et al. noted a remarkable geographical trend, with Denmark having an incidence about twice that of Finland.

Summary

Semen quality has deteriorated in many countries during the last 50 years. The incidence of testicular cancer has been increasing almost invariably worldwide. Increases in the incidences of cryptorchidism and hypospadias have been observed in countries in which longitudinal studies have been performed. However, there are clear regional differences. The prevalence of male breast cancer has been rising and is higher in Denmark than in Finland.

Changes in Male Reproduction in Wildlife. Estrogenic Effects on Developing Animals

Changes in male reproduction in wildlife involve such issues as feminization, demasculinization, reduced fertility, reduced hatchability, reduced viability of offspring, impaired hormone secretion or activity, and altered sexual behavior (*79*). Since it is not possible to review all of the data in detail, the reader is referred to a recent workshop publication titled "Chemically-induced Alterations in Sexual and Functional Development: The Wildlife/Human

Table 1. *(continued)*

	1960–1963	1964–1968	1969–1972	1973–1977	1978–1982	1983–1987
Chinese	–	–	3.3	2.2	1.3	–
Japanese	–	–	–	3	0.6	–
Los Angeles						
White	–	–	–	3.6	4.7	5.8
Spanish	–	–	–	3.1	2.5	3.3
Black	–	–	–	0.9	0.7	0.6
Japanese	–	–	–	1.7	1.9	2.4
Chinese	–	–	–	0.4	0.6	1.9
New York State	2.1	–	2.3	3.2	–	–
New York City	–	–	–	–	2.5	2.9
New York State less city	–	–	–	–	4.1	4.6
Georgia						
Atlanta						
White	–	–	–	4	3.8	–
Black	–	–	–	1	0.5	–
Iowa	–	–	3.7	3.6	4.1	4.4
Louisiana						
New Orleans						
White	–	–	–	3.4	4	3.7
Black	–	–	–	0.6	1.3	0.9
Michigan						
Detroit						
White	–	–	3.2	3.5	4.1	5
Black	–	–	0.9	0.8	1.1	0.9
New Mexico						
Hispanic	–	–	2.8	2.9	3.1	–
Other White	–	–	4.1	4.2	4.2	–
American Indian	–	–	1	1.8	2.1	–
Texas						
El Paso						
Hispanic	–	2.8	2.9	–	–	–
Utah	–	–	3.1	4.7	4	4.4
Washington						
Seattle	–	–	–	4.3	4.7	5.7
Latin America						
Brazil						
Porto Alegre	–	–	–	–	2.5	4.1
Recife	–	–	0.8	–	0.4	–
Sao Paolo	–	–	1.1	1.4	2	–
Columbia						
Cali	2.2	2	1.1	1.5	1.5	1.6
Cuba	–	–	0.2	0.3	–	0.6
Jamaica						
Kingston	0.8	–	0.1	0.6	–	–
Netherlands Antilles	–	–	–	1.1	0.5	–
Costa Rica	–	–	–	–	2.1	1.6
Puerto Rico	0.3	0.7	–	0.5	1	1.2
Asia						
China						
Shanghai	–	–	–	0.9	0.7	0.8
Tianjin	–	–	–	–	0.6	0.5
Hong Kong	–	–	–	1.4	1.2	1.1
India						
Bombay	–	0.9	0.7	0.9	1	1
Bangalore	–	–	–	–	0.7	0.7
Madras	–	–	–	–	0.7	0.6
Poona	–	–	–	1	1	–

(continued)

Connection" (*80*). Many of the reproductive disorders listed above have been associated with xenoestrogenic effects on the fetus. It has usually not been possible to ascribe lowered reproductive success and signs of feminization and demasculinization in wildlife to a single agent; in these cases, chemical analyses of specimens have revealed the presence of multiple compounds, some of which are known to have hormonelike activity. There is experimental evidence that xenoestrogens act cumulatively, i.e., 10 compounds administered simultaneously, each one at 1/10 of their effective dose, resulted in a potent estrogenic response (*81*). Cumulative exposure to nongonadal estrogens is easily documented in male birds, amphibians, and fish by measuring plasma vitellogenin levels.

Gastropod Species

Pseudohermaphroditism or imposex—females developing male characteristics—in marine gastropod species has been reported worldwide: the northeastern United States (*82*), the United Kingdom (*83*), Alaska (*84*), and Southeast Asia (*85*). This phenomenon is caused by tributyltin (TBT) compounds leached from marine antifouling paints used on ships, boats, and mariculture pen nets (*83*). Very low concentrations (1 ng/liter) of TBT-derived tin are effective in induction of imposex (*83*), and marine areas averaging 6 to 8 ng/liter of TBT suffer reproductive failure and local extinction due to female sterility (*86*). The use of TBT was restricted in the 1980s, and recovery of some species has been reported after that (*87*). However, imposex still remains a problem for gastropod species in several marine areas. The mechanism of action of TBT is unknown. The effect mimics that of an antiestrogen, and therefore presents an example of the drastic hormonelike effect of a xenobiotic threatening the existence of several species, even though higher species seem to be far less sensitive to TBT.

Reptiles

Lake Apopka, Florida, was extensively polluted with dicofol (*86*), 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) (and its metabolites 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane [DDD], 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene [DDE] and chloro-DDT), and sulfuric acid spilled accidentally from a chemical company in 1980. Further contamination of the lake by agricultural sewage dumping has made this lake one of Florida's most polluted

Table 1. *(continued)*

	1960–1963	1964–1968	1969–1972	1973–1977	1978–1982	1983–1987
Israel						
All Jews	–	1.5	1.6	1.9	2.2	2.2
Born in Israel	–	2.2	1.3	2.1	1.8	2.7
Born Europe/America	–	2	2.6	2.7	3.8	3.4
Born Africa/Asia	–	0.8	0.8	1	4.9	0.8
Non-Jews	–	0.8	0.2	0.8	0.7	1.1
Total population	1.6	–	–	–	–	–
Japan						
Hiroshima	–	–	–	–	1.7	–
Miyagi	0.6	0.4	0.8	0.8	1	1.3
Urban	–	–	–	1	1	–
Rural	–	–	–	0.5	0.9	–
Okayama	–	1.2	1.2	–	–	–
Nagasaki	–	–	–	1.1	0.8	1.9
Osaka	–	–	0.7	0.8	1.4	1.4
Urban	–	–	–	0.8	–	–
Rural	–	–	–	0.5	–	–
Kuwait						
Kuwaitis	–	–	–	–	1.1	0.8
Non-Kuwaitis	–	–	–	–	1.3	0.6
Singapore						
Indian	–	–	0.5	0.7	0.6	1.2
Malay	–	–	0.3	1.2	0.4	1.3
Chinese	0.5	–	0.9	0.8	0.9	0.9
Pacific Region						
Australia						
Capital Territory	–	–	–	–	4.1	4.2
New South Wales	–	–	–	3	3.5	3.7
Urban	–	–	–	2.9	3.7	–
Rural	–	–	–	3.1	2.9	–
South	–	–	–	3.2	3.4	3.4
Tasmania	–	–	–	–	3.8	5
Victoria	–	–	–	–	4.2	4.2
Western	–	–	–	–	4	3.6
New Zealand	3.5	–	–	–	–	–
Pacific Polynesian Islands	–	–	–	–	1.8	–
Maori	–	3.4	4.3	4.3	7.9	6
Non-Maori	–	–	3.7	4.5	5.3	5.3
European	–	3.2	–	–	–	–
Hawaii						
All groups	2.2	–	–	–	–	–
Caucasian	3.1	2.9	3.3	5	4.2	5.4
Japanese	1.4	1.2	0.3	1.1	1.4	2.9
Hawaiian ethnic	3.3	2.9	3	3.9	3.3	3.6
Filippino	–	0.2	0.3	0.2	0.3	–
Chinese	–	0.7	2.2	0.5	1.2	7.1

[a]Based on data from IARC (*318–323*).

wetlands. A number of these pollutants are known to have estrogenic or endocrine-disrupting effects (*89*). The chemical spill was followed in the subsequent 3 years by a significant decline in the number of juvenile alligators, whereas alligator populations elsewhere were increasing or stable at the same time (*90*). The population decline was associated with reproductive disorders that were hypothesized to be caused by endocrine-disrupting xenobiotics (*91*). There are extensive data supporting the hypothesis (*92,93*): female alligators from Lake Apopka had plasma estradiol concentrations 2 times that of normal females from the control lake, Lake Woodruff. The females exhibited abnormal ovarian morphology. Likewise, in males abnormal germ cells were observed in the testes. Furthermore, Lake Apopka male alligators had abnormally small phalli. Basal and luteinizing hormone (LH)-stimulated plasma testosterone concentrations of male juvenile alligators in Lake Apopka were significantly lower than those of Lake Woodruff males, equaling those of females. The plasma estrogen concentrations of male alligators from these two lakes did not differ. However, testes from Lake Apopka alligators produced significantly more estradiol *in vitro* than testes from control alligators; testosterone production was similar (*93*). The discordance in the *in vitro* and *in vivo* findings suggests additional significant differences in steroid metabolism and liver function between the alligators from these two lakes, emphasizing the complexity of environmental influences. Reptiles have temperature-sensitive sex determination that can be altered by estrogen treatment (*94*). This has been demonstrated both in alligators (*95*) and turtles (*96*). The data from Lake Apopka suggested that the gonads of alligators had been permanently modified, altering steroidogenesis and inhibiting normal sexual maturation (*91,97,98*).

A recent study (*99*) has demonstrated that a number of PCB metabolites are capable of acting as synthetic estrogens. As with crocodilians, many turtles exhibit environmental sex determination so that the temperature at which the egg is incubated determines the sex of the offspring (*97*). Turtle eggs incubated at 26°C produce 100% males. However, if eggs incubated at a male-producing temperature are "painted" with either one of two PCB metabolites (2′,4′,6′-trichloro-4-biphenylol or 2′,3′,4′,5′-tetrachloro-4-biphenylol) sex reversal occurs as if the eggs were treated with the natural estrogen, 17β-estradiol (*99*). Interestingly, if eggs are treated with a dose of both compounds (100 µg of each)—a dose that produced a small percentage of sex-reversed turtles (20 and 0%, respectively)—they act synergistically, producing sex reversal in 80% of the eggs treated. Thus, animals that should have developed as males are modified so that their internal and external morphology is that of a normal female. It is unknown if this sex reversal produces fertile adult females.

Fish

Modifications in reproductive functioning of male fish living downstream from kraft pulp mills have been well documented. For example, white sucker (*Catostomis commersoni*) collected from sites receiving primary effluent exhibited delayed maturation, smaller gonads, and an absence of secondary

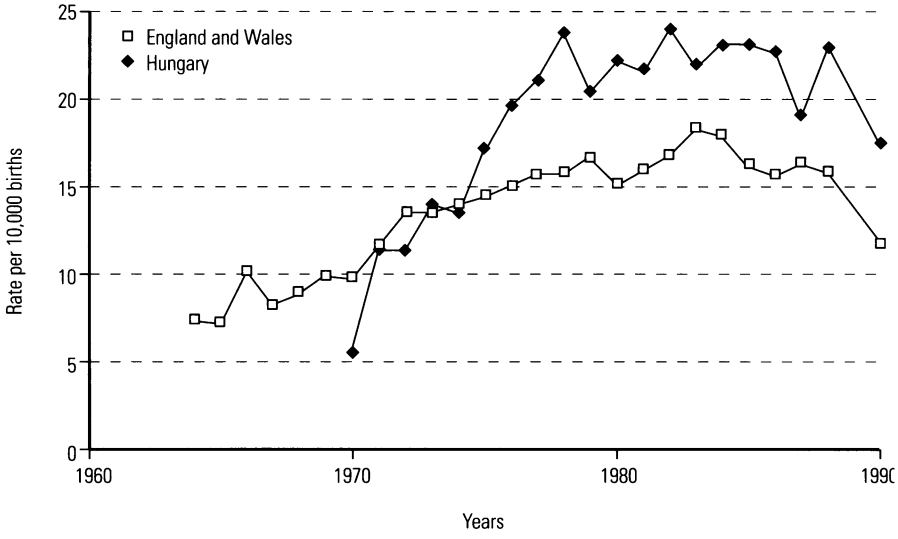


Figure 6. Prevalence of hypospadias at birth (rate per 10,000 births) in England and Wales, and Hungary. Based on data from WHO (*67*).

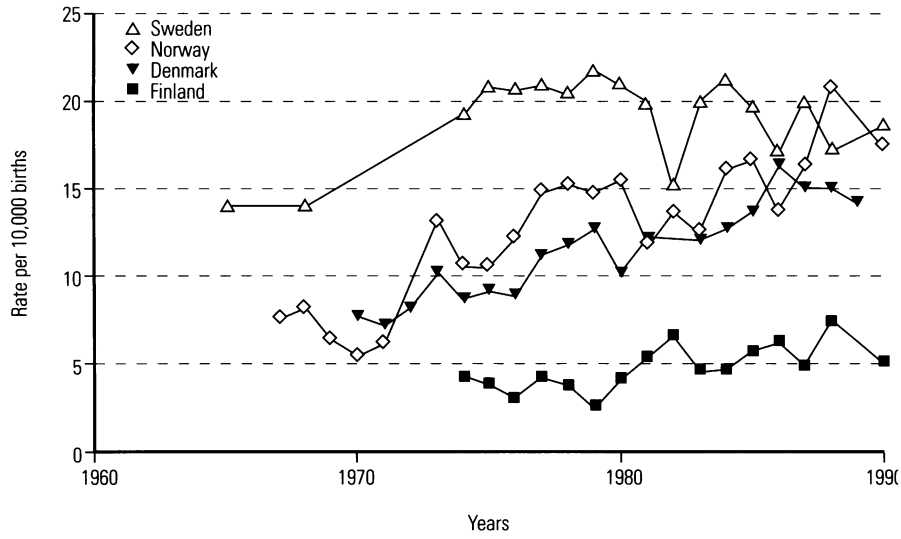


Figure 7. Prevalence of hypospadias at birth (rate per 10,000 births) in the Scandinavian countries. Based on data from WHO (*67*).

sex characteristics (*100*). Furthermore, these males had significantly reduced serum testosterone concentrations and dysfunctional hypothalamic control of the pituitary–gonadal axis. A similar reduction in gonad size was reported for both male and female perch (*Perca fluviatilis*) inhabiting sites receiving primary effluent from a kraft mill on the coast of the Gulf of Bothnia (*101*). The effects observed in these perch were graded along the pollution gradient (2–8 km) from the mill. Masculinization and behavioral changes of female mosquito fish (*Gambusia affinis*) were observed in Florida rivers downstream from paper and pulp mills (*102*). Laboratory studies corroborated the hypothesis that kraft mill effluents containing large amounts of the plant steroids sitosterol and stigmastanol caused the changes (*103*). However, the etiologic agents in paper-mill effluents have not been identified unequivocally. Other effects of paper-mill effluents on fish have been reviewed by Owens (*104*) and Leatherland (*105*).

The effect of PCB exposure on testicular function in fish also has been examined. In the Atlantic cod (*Gadus morhua*), testicular steroidogenesis is disrupted by dietary exposure to PCBs (*106*). Additionally, Freeman et al. (*107*) observed that cod exposed to PCBs *in vivo* exhibited increased metabolism of steroid hormones *in vitro* by kidney and liver tissues. Further, dietary exposure of juvenile males to PCBs precluded the rise in plasma testosterone concentrations associated with sexual maturity. These data suggest that PCBs modify both testicular androgen synthesis and steroid utilization/degradation in peripheral tissues. Exposure to crude oil also induced a decrease in plasma testosterone concentrations in the winter flounder, *Pseudopleuronectus americanus* (*108*).

Organochlorines have also been implicated in a number of developmental and reproductive abnormalities in fish living in the Laurentian Great Lakes of North America. Male coho salmon (*Oncorhynchus kisutch*) living in Lake Erie exhibit a number of abnormalities, including decreased fertility, lower plasma concentrations of gonadotropins and steroids (testosterone, 11-ketotestosterone), poor expression of secondary sex characteristics, and high precocious sexual maturation (*109,110*). It is unknown at this time if the above deficiencies are all related or represent different effects. It is hypothesized that some may be due to modifications of the developing embryo (organizational effects), whereas others may be due to disruptive activational events in adults.

The observation of an increased prevalence of hermaphroditism in fish in sewage treatment water (STW) lagoons in England and Wales initiated a series of studies examining environmental estrogens using a bioassay involving vitellogenin synthesis in STW-exposed rainbow trout (*Oncorhynchus mykiss*) (*111,112*). Vitellogenin is produced in the liver under estrogen control by female fish for the growth of ova (*113*). Males produce it only after exogenous estrogen treatment (*114*). Vitellogenin production by male fish can therefore be used as a biomarker of environmental estrogenic activity. STW exposure of caged rainbow trout induced increases of 500- to 100,000-fold in plasma vitellogenin concentration, and males were shown to achieve vitellogenin levels almost as high as females, indicating the contamination of water by estrogenic compounds (*112*). *In vitro* studies with trout hepatocytes established a dose–response relationship with estrogen exposure and vitellogenesis (*111*). Using this culture system, it was demonstrated that degradation products of several alkylphenol–polyethoxylates, a major

group of surfactants present in sewage, are estrogenic to fish (*111*). Their effect on trout hepatocytes could be blocked with an antiestrogen, Tamoxifen, demonstrating that the compounds act through the estrogen receptor. Although the estrogenic activity of individual xenobiotics was low, their effects may be additive in nature.

Birds

Feminization of gulls and terns in several locations along the Pacific coast of the United States has been associated with DDT and DDE pollution (*115,116*). Fry and Toone (*115*) demonstrated the feminizing capacity of some DDT compounds by injecting gull eggs. Feminization leads to a skewed male/female ratio, which is known to increase female–female pairing. Supernormal clutches, i.e., five to six gull eggs per nest instead of the normal three, are often laid after this type of pairing, and the fertility of the eggs is poor (*117*). There is some controversy as to whether feminization of males or differential mortality of males resulted in a skewed sex ratio (*118*). Fox (*86*) considered both the feminization of males and their increased mortality, when compared to females, as possible reasons for the female–female pairing and supranormal clutches in the gulls.

Mammals

Although the mechanism is unknown at this time, elevated PCBs and DDE concentrations are associated with a decrease in plasma testosterone concentrations in Dall's porpoises, *Phocoenoides dalli* (*119*). Testicular steroidogenesis *in vitro* has been studied in the gray seal (*Halichoerus grypus*) in association with exposure to methyl mercury (MeHg), cadmium (Cd), arsenic (As), selenium (Se), and a PCB mixture (Aroclor 1254). All contaminants except As and Se, stimulated testosterone synthesis *in vitro* from seal testicular tissue (*120*). The mechanism by which this stimulation occurs is unknown. Gonadal steroidogenesis is not the only target for PCBs, as a number of PCB metabolites have been shown to decrease thyroid function *in vivo* in the common seal, *Phoca vitulina* (*121*).

The majority of the remaining (approximately 35 individuals) endangered Florida panthers (*Felis concolor coryi*) exhibit a number of developmental abnormalities and reproductive defects (*122*). Specifically, males ($n = 12$) showed low ejaculate volume, low sperm concentrations ($3–15 \times 10^6$ sperm/ml semen), poor sperm motility, and a very high proportion (92.9%) of sperm with morphological abnormalities (*123*). Cryptorchidism (both uni- and bilateral) has increased exponentially in male cubs since 1975 so that today >90% of the male population exhibits this phenomenon (eight of nine cubs born since 1985). Male sterility may be a problem as well. Female panthers ($n = 3$) have high body burdens of various contaminants including *p,p'*-DDE (5.45–57.65 mg/g lipid fresh weight), PCBs (7.32–27.06 mg/g lipid fresh weight), oxychlordane (< 0.0098–2.00 mg/g lipid fresh weight) and *trans*-nonachlor (< 0.0098–4.82 mg/g lipid fresh weight) (*122*). Panthers also have elevated tissue levels of mercury, methoxychlor, and other lipophilic organochlorine compounds. These contaminants are derived primarily from their major food item, the raccoon (*124*). The reproductive abnormalities described above were suggested to be due to the contamination of mothers by endocrine-disrupting environmental xenobiotics rather than to problems associated with inbreeding (*122*).

Summary

Reproductive disorders in gastropod species, reptiles, fish, birds, and mammals have been associated with environmental factors. Several of the disorders, such as sex reversal in reptiles and vitellogenin production by male fish, may result from estrogenic action of chemicals in the environment. Fewer data are available concerning the mammals. However, some endangered species such as Florida panthers that are exposed to estrogenic and/or other endocrine-disrupting contaminants show reproductive disorders comparable to those found in the human.

Sexual Differentiation and the Physiological Role of Estrogens

Sexual Differentiation

Sexual differentiation occurs during the first trimester of human pregnancy (*125*). An indifferent gonad develops into a testis under the influence of the *SRY* gene on the Y chromosome. In addition to *SRY*, there are several downstream effectors and autosomal genes (e.g., *SOX9* and *SF-1*) that are required for normal differentiation of the testis (*126,127*). Sertoli cells in the newly differentiated testis produce Müllerian inhibiting substance (MIS), which induces regression of the Müllerian ducts that would otherwise develop into the oviducts, uterus, and upper part of the vagina. Sertoli cells also regulate development and early function of the Leydig cells that secrete testosterone to promote differentiation of the embryonic Wolffian ducts into the male accessory sex organs, epididymides, seminal vesicles, and vasa deferentia. Masculinization of the external genitalia and the rest of the body, except the brain, is also controlled by androgens and occurs after conversion of testosterone from the testis into 5α-dihydrotestosterone in the target tissue. Female reproductive organs develop in the absence of *SRY* and thereby in the absence of the testis (*128*). The female developmental pattern seems to be a genetic default pathway, and it is largely independent of hormonal regulation. Disturbances in sexual differentiation occur when factors in the male developmental cascade go wrong or when the genetic female is exposed to an elevated plasma–androgen concentration. In the first instance, a genetic male will develop a female phenotype and in the latter case a female will be virilized.

Disorders of Genital Development and Testicular Malignancy

The association between disorders of genital development and sexual differentiation and gonadal malignancy has been observed since the beginning of this century and is now well established (*129–131*). The most frequent abnormality leading to neoplasia is gonadal dysgenesis with the presence of Y chromosomal material; other disorders include true hermaphroditism and androgen-insensitivity syndrome. Although the general prevalence of disorders of sex differentiation is low, the high incidence of germ cell tumors makes the intersex gonad a good model for the study of factors involved in the pathogenesis of germ-cell neoplasia. Malignant growth frequently appears in the intersex gonad in early childhood, thus suggesting that the carcinogenic process begins *in utero*. The intersex syndrome comprises a variety of genetic disorders, as different from each other as, for example, XY/XO mosaicism and a mutation in the androgen receptor gene. The phenomenon of heterogeneous genetic defects leading to a common result, malignancy of germ cells, indicates that any disruption of early gonadal development may render the germ cells susceptible to neoplastic transformation by yet unknown mechanisms. There are some hypotheses concerning possible mechanisms of neoplastic transformation —e.g., arrested differentiation of immature germ cells (*132*)—or an imbalance in fetal hormonal environment (*133*). Androgen-insensitivity syndrome provides a clue that

the lack of the appropriate inductive hormonal environment may arrest fetal gonadal differentiation and lead to neoplasia later in life. It is possible that high levels of testicular androgens have a protective function during gonadal development; for example, a relative excess of maternal testosterone during early pregnancy was shown in black women compared to a matched white group, providing a possible explanation for the lower incidence of testicular cancer in black men (*134*). There is some experimental evidence that androgens and estrogens may have opposite effects on certain pathways in the developing gonad; e.g., free estrogen may decrease expression of MIS (*135,136*), whereas androgens seem to have a stimulatory effect (*137*).

The Physiological Role of Estrogens in Sexual Differentiation

Estrogens act through a nuclear receptor that is a ligand-activated transcription factor. In addition, steroid hormones may effect the cell membrane. Estrogens are essential in the development of female secondary sexual characteristics and in the female reproductive cycle, fertility, and maintenance of pregnancy. In the developing male, the physiological role(s) of estrogens is unclear, though by analogy to the situation in adulthood, they probably play a role in regulating the differentiation and function of Leydig cells (*138*). The role of estrogen action in embryonic sexual differentiation is controversial. In rats and rabbits (*139,140*), estrogen synthesis is activated in male and female embryos at the time of blastocyst implantation in the uterus. Estrogen receptor mRNA can be detected in blastocysts and two-cell stage embryos (*141*). Immunohistochemical studies by Greco et al. (*142*) demonstrated estrogen receptor expression in both male and female mouse gonads on fetal day 13 and 15. The gonads lose their estrogen receptor expression at later ages. These studies suggested a role for estrogens in development of the gonads (*143*). A putative molecular target of estrogens could be the MIS gene that contains a DNA sequence similar to the estrogen response element in the upstream regulatory region (*144*). In contrast, the classical organ ablation studies by Jost (*145*) demonstrated that gonadectomy of the embryo always resulted in a female phenotype. However, maternal and placental estrogens might still have contributed to this developmental pattern.

Defects in the Estrogen Receptor Gene

Recent reports on estrogen receptor gene-deleted mice (*146*) and a male patient with a defective estrogen receptor (*147*) have begun to clarify the possible importance of endogenous estrogens in sexual development. Lubahn et al. (*146*) disrupted the estrogen receptor gene by targeted deletion that resulted in complete estrogen resistance. Both male and female mice survived to adulthood without apparent morphological anomalies. However, females were infertile with hypoplastic uteri and hyperemic ovaries that contained no corpora lutea. Fertility of the males was also decreased. Only 3 of 15 males that paired with normal females produced any offspring, and even those that were initially fertile lost their ability to sire subsequent litters. Testicular weights were low and sperm counts (in the testis and epididymis) were only 10% of control. Weights of seminal vesicles and coagulating gland were normal. These findings suggest that estrogens are necessary but not indispensable for fetal sexual development; i.e., development is overtly normal, but the sexual organs do not reach their normal size and function.

There is only one reported patient case of estrogen resistance—a 28-year-old white male (*147*). This man had incomplete epiphyseal closure and therefore continued linear growth into adulthood despite otherwise normal pubertal development. He was normally masculinized and had normal male genitalia with bilateral descended testes (20 and 25 ml) and a normal-sized prostate gland. His sexual functions were normal including morning erections and nocturnal emissions. However, his semen quality was subnormal: sperm concentration was 25 million/ml and viability 18% (normal values: > 20 million; > 50%, respectively). His serum testosterone concentration was normal, whereas estradiol, estrone, follicle-stimulating hormone (FSH), and LH concentrations were elevated. The elevated gonadotropin concentrations suggest that estrogens play a role in the regulation of gonadotropin secretion in males, and thereby may have several indirect effects. The patient case and the receptor gene-deleted mice demonstrate that a normal male phenotype develops in the absence of estrogen receptor-mediated influences, but semen quality and probably fertility may be compromised as a result.

Overexpression of the Estrogen Receptor

In transgenic mice that overexpress the estrogen receptor, normal differentiation of sexual organs was observed (*148*). However, females in several transgenic lines had fertility problems and their gestational length was significantly prolonged, resulting in loss of litters due to difficulties in parturition. No major fertility problems were reported in the male transgenics. The only abnormalities described in the males (as well as in the females) were hernias of the abdominal wall musculature. This is of interest, since estrogen administration has been reported to induce inguinal hernias in male mice (*149*), and dogs (*150,151*), and there is some indication that inguinal hernias are a risk factor for testicular cancer (*152*). It is not known yet whether transgenic male mice that overexpress the estrogen receptor will develop testicular tumors later in life.

Nonmammalian Vertebrates

Amphibians and birds differ from mammals in their sexual differentiation (*153*). That is, the female phenotype in birds develops under estrogen control, whereas the male phenotype appears in the absence of estrogen (*154*). Exogenous estrogen administration causes sex reversal in male birds and frogs. Some reptiles (crocodilians and some turtles) have temperature-dependent sex determination; for example, in turtles, female hatchlings are produced by incubation of the eggs at a higher temperature than males, but an excess of estrogen causes feminine differentiation also at low temperatures typical of males (*155,156*). Estrogen-induced sex reversal can be used as a biomarker of the estrogenicity of an environmental pollutant, as demonstrated recently for PCBs (*99*). Estrogen receptors are expressed both in female and male chick embryos in the Müllerian ducts (*154*). Interestingly, the left Müllerian duct that develops into an oviduct in females (regresses in male) has higher estrogen binding capacity than the right, which regresses in both sexes (*157*). Treatment of chick embryos with diethylstilbestrol (DES) on day 5 prevented regression of the Müllerian ducts in both sexes (*158*). Similar findings in the mouse are reviewed in the section "Effects of Synthetic Estrogens on the Testis in Animal Models."

Estrogens and Sperm Production Capacity

Sperm production is dependent on permissive actions of FSH and testosterone (and therefore LH). Sperm production capacity depends on the number of Sertoli cells in the seminiferous tubules (which is directly related to the length of the tubules) (*159*), since each Sertoli cell supports a finite number of germ cells (*160*). Sertoli cells proliferate quickly in rats from embryonic day

19 to day 15 after birth, then slow down and cease multiplication approximately on postnatal day 20 (*161,162*). Multiplication is largely dependent on FSH stimulation (*162*). In humans, regulation of Sertoli-cell proliferation may be very similar. Men with hypogonadotropic hypogonadism do not develop normal-sized testes after gonadotropin treatment, which may be a consequence of inadequate Sertoli cell multiplication in early childhood. This hypothesis is supported by findings in the monkey (*163*): Sertoli cells proliferate in the neonatal and infantile period but not during or after puberty. Estrogens suppress gonadotropin production in animals at all ages preceding puberty (*164*). It is hypothesized that this is the case also in humans. Decreased gonadotropin stimulation during the critical developmental phase may result in inadequate Sertoli cell proliferation and small testes (*165*). Specific FSH gene deletion experiments also demonstrated that FSH regulates the size of the testis (TR Kumaar, personal communication). At present it is not known whether the number of Sertoli cells in human testes has decreased and whether this might be a reason for decreased sperm counts.

Summary

Normal masculine differentiation occurs under the influence of the *SRY* gene and several other autosomal genes, and androgens are required for this process. Disorders of gonadal development are frequently associated with testicular germ cell neoplasia. Estrogens act through a specific nuclear receptor. Normal masculine differentiation occurs even in the absence of a functioning estrogen receptor, but the patient with the receptor defect had poor semen quality. Estrogen receptor-deficient male mice were subfertile and few were able to sire one litter. Estrogens are involved in the feedback regulation of gonadotropin secretion, and the suppression of FSH secretion during the period of Sertoli cell proliferation (perinatal period) may result in small testes and a low sperm production capacity in adult life.

Occurrence of Abnormalities in the Reproductive System of the Sons of Women Exposed to Diethylstilbestrol during Pregnancy

Exposure

Diethylstilbestrol (DES) was prescribed to more than five million pregnant women from the late 1940s to the early 1970s to prevent abortions and pregnancy complications (*166*). Dieckmann and co-workers performed a double-blind placebo-controlled study on the therapeutic value of DES during pregnancy in the early 1950s (*167*). DES was given to 840 pregnant women and placebo to 806 controls. Compliance was verified by a dye indicator in the urine during the whole study. The women entered the study between weeks 7 and 20 of pregnancy (the majority during weeks 10–12) and received increasing doses of DES until pregnancy week 35 (5–150 mg/day). This study clearly indicated that the medication was not efficacious in the indications for which it was used (*167*). Instead, in the reanalysis of the material of Dieckmann et al. (*167*), DES was associated with significant increases in abortions, neonatal deaths, and premature births (*168*). When Herbst and co-workers (*169,170*) reported the high incidence of a very rare cancer, clear cell adenocarcinoma of the vagina, in pubertal girls exposed to DES *in utero,* the U.S. Food and Drug Administration (FDA) banned the use of DES during pregnancy. Medical authorities in Europe that had allowed DES use for pregnant women soon followed FDA regulations. In Europe, approximately 200,000 French, more than 150,000 Dutch, 63,000 Czechoslovakian, and 7000 British women were exposed to DES, whereas in the United States 4.8 million women were prescribed DES during pregnancy. In addition, DES was used as an anabolic agent in livestock, and the general population that used dairy products and meat may have been exposed to the hormone via this route to an unknown, and probably variable, extent. Some of the DES-exposed daughters and sons have been followed since the 1970s and a significant number of abnormalities in the structure and function of reproductive organs have been described (*171*).

Structural Anomalies

Structural anomalies of the reproductive organs that are significantly more frequent in DES-exposed male subjects than in controls include meatal stenosis (12.9 vs 1.8%); hypospadias (4.4 vs 0%); epididymal cysts (20.8 vs 4.9%); testicular abnormalities, including hypoplastic testis, cryptorchidism, and capsular induration (11.4 vs 2.9%); and microphallus (4 cases vs 0 cases) (*172–174*). The data of Bibbo and Gill and their co-workers (*173,174*) are based on the follow-up studies of the offspring of mothers who took part in the double-blind study of the effects of DES on pregnancy in 1953 (*167*), and therefore the studies can be considered prospective. There were 308 men exposed to DES and 307 men exposed to placebo included in the study; 31.5% of men exposed to DES had an abnormality of their reproductive tract, whereas only 7.8% of controls had an anomaly (*174*). In the recent follow-up study of these males, it was found that the men who were exposed to DES before week 11 of gestation had twice as high a frequency of genital anomalies than did those who were exposed only later (*175*). This finding indicates the importance of the timing of the exposure (time of organogenesis). In a small study comprising 17 DES-exposed men, 12 nonexposed volunteers, and 11 fertile control men, genital anomalies (varicocele, epididymal cysts, absent testes) were reported in 13 of the DES-exposed subjects, 4 of the volunteers and 4 of the fertile normal controls (*176*). Whitehead and Leiter (*177*) reported genital abnormalities in 29 of 48 men exposed to DES. Hypertrophy and squamous metaplasia of the prostatic utricle was found more frequently in aborted male fetuses that had been exposed to DES than in nonexposed controls (*178*), suggesting that DES-exposed males may have an increased risk of prostatic hyperplasia and/or cancer when aging. The data connecting DES exposure to several structural abnormalities of the male reproductive tract are convincing and leave little space for speculation on confounding factors. However, no association was found between first-trimester exposure to sex hormones, other than DES, and external genital abnormalities in a recent metaanalysis of 14 studies (*179*). In a large cryptorchidism study, no association between the disorder and exposure to estrogens during the pregnancy could be found (*180*).

Semen Quality

Gill et al. (*181*) studied semen samples from 88 men exposed to DES and 85 men exposed to placebo, who were offspring of the mothers from the 1953 study performed by Dieckmann and co-workers (*167*). Sperm concentration of men exposed to DES was significantly lower than in the controls (83 million/ml vs 123 million/ml, $p < 0.02$). There was no difference in semen volume, whereas the total sperm count, sperm motility grade, the total number of motile sperm, the percentage of sperm with normal morphology, and the quality score

were all statistically lower in men exposed to DES. Azoospermia was found only in men exposed to DES, and 20.5% compared to 3.5% of men who received placebo had a sperm concentration in semen of less than 20 million/ml. The groups did not differ in their testosterone, FSH, or LH levels (*173*). In a later study on the same men (20 controls declined to participate), sperm concentrations still differed significantly, whereas other semen characteristics were similar between the groups (*182*). Similar results were obtained in another study (*176*) in which the mean sperm concentration of men exposed to DES was 66.4 million/ml compared to 101.7 million/ml in normal volunteers ($p < 0.05$). In this study, the zona-free hamster egg penetration assay was also performed: sperm from 14 of 17 men exposed to DES failed to penetrate more than 14% of the eggs (which is the reference value for the normal fertility range), suggesting infertility, whereas only 2 of 12 unexposed volunteers and none of 11 fertile normal controls had an abnormal test result. In the study performed by Whitehead and Leiter (*177*), only 33% of the men exposed to DES had normal semen quality. However, Andonian and Kessler (*183*) found no difference in semen quality between 24 men exposed to DES and 24 age-matched control men. Again, the large 1953 study population that has been followed prospectively appears the most valid for evaluation of semen quality. On the basis of that finding, DES exposure resulted in a significant decrease in semen quality.

Semen quality and fertility are not in direct correlation. In the latest follow-up study of the Dieckmann cohort, no difference in the fertility between men exposed to DES and their controls were found (*175*). This is compatible with the earlier findings (*181*) that the majority of the men exposed to DES had sperm concentrations well above the limit at which fertility is supposed to be disturbed (20 million/ml), although the mean sperm concentrations of exposed men were lower than those of controls.

Testicular Cancer

There is no conclusive evidence to indicate an increased risk of testicular cancer in men exposed to DES (*184*), although the incidence of cryptorchidism is a well-known risk factor for testicular cancer and has been observed more frequently in this group (*171*). Two patient cases with seminoma in men exposed to DES have been reported (*185*), but epidemiological studies have failed to show a statistically significant relationship between DES exposure and testicular cancer. There have been a few case–control studies that evaluated prenatal hormonal risk factors for testicular cancer (*186–191*). In the first study (*186*), 131 testicular cancer patients, under age 40, and their matched controls were analyzed. In 6 cases of cancer the mothers had been treated with hormones during pregnancy, whereas only one mother of the control cases had received any hormones. The difference was not statistically significant, but if another factor, nausea, was combined with hormone treatment, they formed a significant risk factor (relative risk 4.33).

In the case–control study of Depue et al. (*187*), 108 testicular cancer patients, under age 30, were studied. Mothers of 9 cancer patients had been treated with hormones (2 with DES, 1 with estrogen, 1 with progestin, and 5 had pregnancy tests consisting of a single injection of an estrogen–progestin preparation), whereas 2 controls had either estrogen treatment or a pregnancy test. The relative risk (8.00) was significantly increased in the men exposed to hormones ($p = 0.02$). However, the exposures were very heterogeneous, and single pregnancy tests can hardly be compared to long-term DES treatment.

In a similar study comprising 202 cancer cases and 206 controls, Brown et al. (*189*) found no excess risk associated with the use of hormones during pregnancy: mothers of 4 cancer patients and 5 control mothers had received hormone treatment. Two mothers in each group had been treated with DES, 1 control with estrogen, 1 case with progesterone, 1 in each group had a hormone pregnancy test, and 1 control had an unidentified hormone treatment. However, it should be noted that 19 mothers in this study were medicated for bleeding problems, but only 2 (both case mothers) mentioned a specific hormone used; 13 of the treated were case mothers and 6 were control mothers.

In a case–control study of 273 testicular cancer patients from northern California (*190*), no association was found with the mother's hormone exposure or DES exposure. Mothers of 9 cases and 10 controls had been treated with hormones (odds ratio 0.9). Four of the case mothers and 2 control mothers were exposed to DES.

The case-control study of Schottenfeld et al. (*188*) was based on questionnaires received from 190 testicular cancer patients (The Sloan Kettering Cancer Hospital, New York), 166 hospital controls, and 143 neighborhood controls. There was no statistically significant association between hormone treatment and cancer: 5.8% ($n = 11$) of cases had been exposed to DES or other hormones, whereas 2.1% ($n = 3$) of the neighborhood controls and 2.5% ($n = 4$) of the hospital controls had received exogenous hormones. Similarly, a case–control study of 79 testicular cancer patients from the Connecticut Tumor Registry failed to show any increased cancer risk in men exposed to DES (*191*).

The studies above have been described in detail because they illustrate two major problems. First, DES treatment may have been initiated at various times during pregnancy; therefore, the presumed critical period during which adverse effects of estrogens might occur may have been missed in some of these studies. Second, the investigated populations of testicular cancer patients have been too small to determine if a significant difference truly exists between DES-exposed and nonexposed men. When we combined the data presented above in a metaanalysis, a marginally significant increase in testicular cancer incidence for the individuals exposed to hormones (including all hormones) was found; Mantel-Haenszel estimates of the common odds ratio was 2.1 with 95% confidence intervals of 1.3 to 3.3. Exposure to DES was a significant risk factor for testicular cancer on the basis of our metaanalysis: odds ratio was 2.6 with 95% confidence limits of 1.1 to 6.1. It would be most important to obtain additional information on the incidence of testicular cancer in men born to mothers who participated in the double-blind, placebo-controlled DES trial in the 1950s (*167*).

Summary

Exposure to DES during pregnancy results in an increased risk for several male reproductive disorders, such as cryptorchidism, urethral abnormalities, epididymal cysts, and testicular hypoplasia. In addition, the semen quality of DES sons is worse than that of controls. Incidence of testicular cancer is approximately doubled among DES sons compared to the general population, but whether this represents a true increase of the cancer risk is equivocal.

Effects of Synthetic Estrogens on the Testis in Animal Models

Synthetic estrogens, such as DES, ethinyl estradiol, and estradiol benzoate have been

thoroughly studied in several animal models because of their pharmaceutical applications. There are comprehensive reviews covering this topic (*164,192,193*).

Mechanism of Action

The effects of estrogens depend on the dose, time, and probably the duration of exposure. Estrogens also act at several levels in the reproductive system, i.e., they influence specific neuronal areas in the brain, they modulate gonadotropin secretion from the pituitary gland, and they directly affect the reproductive organs. Estrogens probably exert most or all of their effects through a specific receptor; such receptors are present in the brain, pituitary, gonads, and accessory sex organs at one or another time during fetal, prepubertal, or adult life (*143*). However, the precise localization and temporal expression of estrogen receptors during differentiation and development of the testis and male reproductive tract are poorly described and further, more definitive, studies are needed. The effects of DES are not unique to this compound but are probably shared by all estrogens (*164*). Many of the synthetic estrogens are more effective in lower doses than endogenous estradiol because they are not bound by sex hormone-binding globulin (SHBG), which normally binds approximately 95% of circulating estradiol, rendering it biologically inactive. Estrogens are metabolized rapidly in the testes, e.g., by specific sulfotransferases, after which they cannot bind to their receptor (*194*). If the active center of the enzyme is occupied by a xenobiotic, metabolism of endogenous estrogens may be disturbed and high levels of active hormone may be available. This is particularly important during fetal development when the levels of ambient estrogens are high.

Adverse Effects of Neonatal Estrogen Treatment

Long-lasting suppression of spermatogenesis and atrophy of reproductive organs in neonatally estrogen-treated rats and mice have been described by many workers since the 1950s (*164*). A single injection of estradiol benzoate (250 µg on day 5) resulted in a marked delay of the onset of puberty in rats (*195*). When mice were treated with repeated doses of estradiol on days 1 to 5 after birth, the testes were irreversibly damaged, and subsequent treatment with testosterone and gonadotropins failed to maintain spermatogenesis in the majority of these estrogenized mice (*196*). These two studies are cited because they indicate that estrogens given neonatally act directly on both the testes and the pituitary gland. It is noteworthy that the neonatal period in rodents corresponds in many ways to the second and third trimesters of pregnancy in the human.

Adverse Effects of Prenatal Estrogen Treatment

Prenatal (day 11 and 12 postcoitum) exposure of mice from the Sv-Sl CP strain (a strain in which the males are susceptible to testicular teratomas) to ethinyl estradiol resulted in an increased incidence of cryptorchidism ($p = 0.0001$), and 19 of 224 exposed male animals developed testicular teratoma compared to 4 out of 107 controls (the odds ratio of 2.4 was not significantly different) (*197*).

McLachlan and co-workers have performed a large series of studies on the effects of prenatal exposure of mice to DES (*192,193*). In most of the studies, pregnant mice were treated with 0.01, 1, 10, or 100 µg/kg/day DES or corn oil on days 9 to 16 of pregnancy (time of sexual differentiation). The high doses are closely equivalent to those used for pregnant women (*173*). Male offspring from these pregnancies suffered from the same structural and functional anomalies reported in men exposed to DES, i.e., epididymal cysts, cellular atypia in the prostate, cryptorchidism, testicular hypoplasia, poor semen quality, and subfertility (*192*). In addition, Sertoli-cell hyperplasia, interstitial testicular tumors, squamous metaplasia of the seminal vesicles, and rete testis adenocarcinoma were found frequently in the male offspring of mice exposed to DES during pregnancy (*198*). The analogy between the findings in the human and the mouse illustrates how informative and relevant the results from animal studies are.

Estrogen Effects on the Müllerian Ducts

Müllerian inhibiting substance secreted by Sertoli cells is responsible for regression of the Müllerian ducts. Analysis of male mouse embryos exposed to DES revealed delayed and incomplete regression of the Müllerian ducts (*192*). *In vitro* organ culture experiments verified the inhibitory action of DES on Müllerian duct regression (*136*). Estrogen receptors were found both in the Müllerian ducts and Sertoli cells at the time of regression (*143*). Estrogens could either affect the Müllerian ducts directly or influence the expression of the *MIS* gene in the Sertoli cells. Some of the structural abnormalities observed after birth may arise as a result of incomplete regression of the Müllerian ducts (*198*).

Estrogen Effects on the Developing Testis

Reports on various experimental animals (e.g., sheep, rats, mice) describe how exposure to exogenous estrogens during the neonatal period causes drastic reductions in the secretion of FSH from the pituitary gland and the presumption is that similar effects would occur before birth (*199*). As FSH plays a vital role in controlling multiplication of Sertoli cells at this time (*138*) the prediction would be that estrogen-induced suppression of FSH levels would lead to a slower rate of Sertoli cell multiplication. As the number of Sertoli cells formed in fetal/neonatal life is an important factor influencing the maximum level of sperm production in adult life, the consequences of such a change in terms of sperm counts is obvious; moreover, such an effect is irreversible once Sertoli cell multiplication stops in early postnatal life. There is abundant evidence from man (hypogonadotropic hypogonadism) and from animal species that suppression of FSH levels in early postnatal life results in just such changes [reviewed by Sharpe (*138*)]. Recent evidence from the fetal sheep (*165*) also shows that suppression of FSH secretion in the fetal male during the second half of gestation results at birth in testes that contain 40% fewer Sertoli cells than occurs in control animals.

It is therefore hypothesized that prolonged exposure of the developing male, during both fetal and postnatal life, to exogenous estrogens (perhaps even at low levels) could reduce Sertoli cell number and thus reduce sperm output (and sperm counts) in adult life. Experiments involving exposure of rats to various xenoestrogens during the period of Sertoli cell multiplication have shown that in adult life such exposure results in small (8–12%) but highly significant reductions in testis size and a corresponding decrease in daily sperm production (*200*). These effects have been achieved after exposure to relatively low levels of the chemicals (alkylphenols, phthalates; 1 mg/liter in drinking water of pregnant rats) under test. For example, butylbenzyl phthalate has been found to occur in butter and margarine at concentrations as high as 47.8 mg/kg (*201*). Such findings suggest that there is the theoretical possibility that human exposure to

such chemicals might have contributed to the decline in sperm counts in men described earlier.

Summary

Diethylstilbestrol treatment of experimental animals *in utero* results in increased incidence of cryptorchidism; urethral abnormalities; testicular hypoplasia; poor semen quality; and infertility, abnormalities in accessory sex organs, rete testis adenocarcinoma, interstitial cell hyperplasia, and tumors. Thus, the outcome of DES exposure of experimental animals is highly analogous to the findings in humans. Recent data in the rat suggest that perinatal exposure to xenoestrogens, such as butylbenzyl phthalate, results in decreased size of the testes and daily sperm production in adult life.

Environmental Chemicals with Known Estrogenic Effects

Estrogenic effects are not restricted to a small group of therapeutic agents but appear in several groups of compounds that are used daily in industry, agriculture, or in the home (*79,80,202–204*). The major groups of environmental chemicals, such as organochlorine pesticides, PCBs, dioxins, alkylphenol polyethoxylates, phytoestrogens, and other xenoestrogens, currently known to have estrogenic effects in vertebrates or in assays *in vitro* are discussed here. A major problem is the determination of those chemicals that are estrogenic (or otherwise endocrine-disrupting, i.e., disturbing normal endocrine homeostasis). At present, tens of thousands of man-made chemicals are being used, yet the effects on the endocrine system have been studied for only a few of these. The estrogenic activity of the majority of chemicals (e.g., alkylphenols, phthalate esters, bisphenol-A) has been detected by accident, not by intent, although recently some screening of chemicals used in large volumes has been attempted (*204*). Hence, it is highly possible that other estrogenic chemicals remain unidentified. However, it should be remembered that many of the chemicals to which man is exposed have been tested (often in two- or three-generation studies) before being approved for use; and hence, if any of these chemicals were a strong estrogen, this would probably have been discovered. This is especially the case for chemicals that are currently approved for use in food production, such as food additives and pesticides, and for new chemicals that have been produced in large amounts from the early 1980s in the European Union (EU). The current legislation demands extensive documentation for safety by regulatory agencies before a chemical can be used in foods or commercial products. However, many chemicals were introduced before these strict regulations were enforced. Thus, the present situation is that man and wildlife are exposed to a very wide range of chemicals. For the majority of these we do not know whether they are estrogenic, whether their effects are additive, or even what the true exposure to these chemicals is.

A xenoestrogen can induce its estrogenic effect in multiple ways: it may act directly through estrogen receptors, or it may disturb estrogen metabolism, thus increasing the levels of the endogenously produced ligand. Different estrogenic and antiestrogenic ligands form functionally different complexes with the estrogen receptor, and their transcriptional effects depend on the cell type and promoter (*205*). Thus, the same compound may potentially have an estrogenic effect in one system or at one concentration, and an antiestrogenic effect in another system or at another concentration. Furthermore, effects of many compounds influencing other hormone systems (e.g., antiandrogens) may mimic those of estrogens.

A number of chemicals, mainly pesticides and many of which are currently being used, have been implicated as environmental hormones possessing endocrine-disrupting properties (*80*). In the public debate on male reproductive disorders, this has misled many to suppose that all of these chemicals are estrogenic. In fact, many of these compounds have not been adequately tested for estrogenic activity. However, for many others, a large toxicological database exists, including data on reproductive toxicity, effects on steroid-metabolizing enzymes, and effects on hormone-producing tissues. A short summary of the most relevant toxicologic effects known for a number of xenoestrogens and other environmental chemicals that have been implicated as environmental hormones is given in "Appendix A." It also outlines the safety assessment procedures and principles applied world-wide by regulatory agencies.

Below is a short examination of each of the groups of chemicals that are known to be estrogenic.

Organochlorine Pesticides

Organochlorine pesticides include dichlorodiphenylethanes (DDT, DDD, DDE, dicofol, perthane, methoxychlor), cyclodienes (chlordane, oxychlordane, *trans*-nonachlor, heptachlor, heptachlorepoxide, aldrin, and dieldrin), hexachlorobenzene, and hexachlorocyclohexanes (*206*). Many of these, most notably DDT, were used in large quantities until the 1960s when the use of DDT was banned or restricted in Western countries. Hexachlorobenzene, however, was used in the United States until 1985. DDT products are still used widely in many developing countries. Despite restrictions on their use, these compounds are still circulating in the environment because many of them bioaccumulate and become concentrated in body lipids (biomagnify). The breakdown and elimination of these compounds is very slow; therefore, their effects can be persistent, lasting for generations (DDT has a half-life of >60 years in the environment). Long-term exposure to small amounts of organochlorine contaminants leads to the accumulation of considerable burdens in animal and human tissues (*207,208*). It is therefore not the amount of DDT to which a mother is exposed during pregnancy that is critical but rather her lifetime exposure that will determine the level of exposure of the fetus and the breast-fed infant.

Commercial DDT contains several isomers of which *p,p′*-DDT is the most prevalent (75–80%), whereas the proportion of the most estrogenic isomer *o,p′*-DDT is 10 to 25% (*89,209*). The *o,p′*-isomers are less stable than the *p,p′* configurations and are therefore found only in low concentrations in nature (*210*). However, *p,p′*-DDT was also reported to have estrogenic actions both on the rat uterus (*211*) and in the MCF-7 breast cancer cell line (*212*). The estrogenic activity of DDT isomers compared to that of estradiol is very weak (10^3–10^6 times less potent). However, the long half-life and bioaccumulative properties of DDT indicate that levels of human exposure may be sufficient to induce estrogenic effects in certain circumstances. This is particularly true for the period from the 1940s to 1960s when DDT was used widely including in direct application to humans.

Antiandrogenic (demasculinizing) and estrogenic (feminizing) effects often manifest themselves in the same way, although through distinct receptors (*213*). Therefore the recent discovery that *p,p′*-DDE, the main metabolite of DDT in the body, is a potent antiandrogen (*214*) may explain some of the estrogenic effects observed in the environment; many of these effects

may occur due to an antiandrogen activity of a xenobiotic.

Fry and Toone (*115*) induced feminization in male California gulls (*Larus californicus*) by injecting eggs with DDT in amounts that were comparable to those found in seabird eggs in southern California in the late 1960s. A skewed sex ratio in favor of females in large gull populations suggested the possibility of a causal relationship with the estrogenic action of DDT (*86*). The effects of DDT metabolites and dicofol in reptiles have been discussed in the section "Changes in Male Reproduction in Wildlife. Estrogenic Effects on Developing Animals." In mammals, the effects of DDT compounds on male reproductive function are less apparent (*215*).

Methoxychlor is estrogenic in the E-SCREEN assay (*204*). It was also found to be estrogenic *in vivo* in rats (*216*). Methoxychlor or DDT exposure of neonatal rats did not affect male reproductive organ weights in adulthood (*217*), and neither induced epididymal cysts (*218*), which were found frequently in mice exposed to DES (*192*). However, exposure throughout gestation and lactation in rodents resulted in slightly smaller testes and epididymides and in lower sperm counts in male offspring than in controls (*219,220*). It was suggested that the inability of the neonatal rodent to metabolize methoxychlor to its active estrogenic form might explain the discrepancy between these studies (*220*).

Chlorinated cyclodienes induce liver enzymes that hydroxylate testosterone (*221*). Chlordane disturbed spermatogenesis and caused dose-related damage to the testes of mice fed for 30 days with 0.08 mg or 0.25 mg of the active ingredient (*222*). Mating studies of dieldrin-exposed rats suggest male-dependent disturbances in fertility (*223*). In the E-SCREEN assay, chlordane and heptachlor were not estrogenic, but the heptachlor derivative 1-hydroxy chlordane was (*212*). In addition, dieldrin was shown to be estrogenic (*81*).

Hexachlorobenzene was also reported to induce liver enzymes hydroxylating androgens (*221*). Long-term studies have demonstrated liver and kidney anomalies in exposed animals but indicate no effect on fertility (*224*).

Hexachlorocyclohexanes (HCHs) comprise several isomeric forms; these compounds are also called benzene hexachloride (BHC). γ-HCH has the common name lindane and is the most acutely toxic of the isomers (*215*). The most persistent and bioaccumulating isomer is β-HCH, which accounts for 90% of the total HCH found in human milk (*225*). Lindane was reported to have both estrogenic and antiestrogenic effects in female rats (*226*). In male weanling rats fed with β-HCH (0, 2, 10, 50, or 250 mg/kg) for 13 weeks, liver enzyme induction occurred at doses > 2 mg/kg; testis weights decreased at doses > 50 mg/kg; and testicular atrophy resulted from a dose of 250 mg/kg (*227*).

In hamsters, a single injection of the weakly estrogenic chlordecone (Kepone) in neonatal males reduced testicular and epididymal weight (*228*). Estrogenicity of chlordecone was also demonstrated in rats (*229*) and birds (*230*).

Polychlorinated Biphenyls

Polychlorinated biphenyls are industrial chemicals used since 1929 as heat transfer and hydraulic fluids, adhesives, flame retardants, dielectric fluids for capacitors and transformers, and waxes (*231*). PCBs consist of 209 congeners, which are found in different mixtures in commercial products. Before the production of PCBs was banned in the United States in 1977, hundreds of millions of kilograms were produced, and a large proportion of the synthesized product is still in the environment because of bioaccumulation and slow biotransformation.

The biological effects caused by the various congeners differ, not only in potency but also qualitatively. Several non-*ortho*- and mono-*ortho*-substituted PCB congeners induce effects similar to those caused by chlorinated dioxins and dibenzofurans; i.e., the toxicity is probably mediated through interaction with the aryl hydrocarbon (Ah) receptor. Other PCB congeners presumably act by different mechanisms. In addition, there are PCB congeners that are intermediate in this respect; i.e., they elicit a mixed spectrum of effects. Our knowledge of possible interactions between the various groups of PCBs is still very limited (*232*).

Both estrogenic and antiestrogenic effects have been reported for different PCB congeners (*233*). The estrogenic potency appears to depend on the percentage of chlorine: less-chlorinated PCBs (Aroclors 1221, 1232, 1242, and 1248) have estrogenic activity whereas more chlorinated congeners do not (*209*). The stability of the compounds increases with higher chlorination. Less-chlorinated compounds were shown to transfer more readily across the placenta than were the highly chlorinated PCBs (*234*). PCBs are hydroxylated in animals, and these hydroxybiphenyls are quite active as estrogenic compounds [i.e., more than 1/100 of estradiol activity (*235*)]. Antiestrogenic effects have been found in MCF-7 breast cancer cells with 3,3′,4,4′-tetrachloro-biphenyl (a dioxinlike PCB), a form known to bind to the Ah receptor, mediating the effect (*233*). Reproductive failure of seals in the Wadden Sea has been attributed to PCBs (*236*), and has been supported by laboratory studies (*121*). However, this relationship may not necessarily have been a consequence of the estrogenicity of the PCBs.

Dioxins and Furans. Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) consist of 75 and 135 different congeners, respectively (*237*). The most toxic congener is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), commonly referred to as dioxin. These compounds are formed as unwanted by-products in the manufacture of chlorinated hydrocarbons. Other sources include incineration processes, paper and pulp bleaching, and emissions from steel foundries and from motor vehicles (*238*).

Most of the animal studies on dioxins have been performed with TCDD [reviewed by Peterson et al. (*239*)]. Dioxins act through an Ah receptor that is also involved in mediating the antiestrogenic effects of TCDD (*240,241*). However, the role of the Ah receptor has not been established for several of the toxic effects that are found in males exposed to TCDD (*242*). There is considerable literature documenting the toxic effects of dioxins on the male reproductive system (*239,242*). Prenatal and lactational exposure of male rats to TCDD profoundly disturbed the developing male reproductive organs: anogenital distance was shortened, testicular descent was delayed, and the weights of all sex organs were reduced (*243*). Furthermore, spermatogenesis was inhibited, sexual behavior was feminized and demasculinized, and the regulation of LH secretion was feminized (*244–246*). Perinatal suppression of plasma testosterone concentrations appeared to be involved in the changes described. The effects were elicited by a single maternal oral dose of TCDD on day 15 of pregnancy [ED_{50} approximately 0.16 μg/kg; at this dose, TCDD had no discernible effect on the mother (*242*)]. Most of the effects were found at the lowest dose level tested (0.064 μg/kg), whereas in other studies, reproductive toxicity had been observed with doses of < 0.001 μg/kg/day (*247,248*). The mechanism of action of

dioxins and furans may not be primarily estrogenic or antiestrogenic (e.g., antiestrogenic effects of TCDD cannot be reversed by high estrogen concentrations), but it is certainly a hormonelike effect. This necessitates the close surveillance of humans exposed to TCDD.

Alkylphenol Polyethoxylates

Alkylphenols and related compounds are present in surface waters and aquatic sediments (*249–251*). They are products of the microbial breakdown of alkylphenol polyethoxylates (APEs) that are widely used in industrial surfactants (*250,251*). These effective, nonionic surfactants are used in detergents, paints, herbicides, pesticides, and cosmetics, to name a few major groups of products. Over 300 million kilograms of APEs are produced annually. After sewage treatment, approximately 60% of the APEs are released into the aquatic environment as short-chain APEs (e.g., nonylphenoldiethoxylate [NP2EO]), alkylphenol carboxylic acids (e.g., nonylphenoxycarboxylic acid [NP1EC]), and alkylphenols (e.g., nonylphenol [NP]; and octylphenol [OP]) (*252–254*). Alkylphenols are relatively persistent and bioaccumulate in the lipids of living organisms (*255,256*).

NP and OP were shown to be estrogenic both *in vivo* and *in vitro* in mammalian systems (*202,257*). The effects were estrogen receptor mediated. OP was more potent than NP, reaching approximately 1/1000 of the potency of estradiol. In the trout hepatocyte assay, many other APEs were also shown to be weakly estrogenic (*111*).

Phytoestrogens

Phytoestrogens are natural compounds present in plants and are ingested daily in milligram quantities. The active substances are isoflavones (genistein and daidzen) and coumestans (coumesterol) (*258*). Fungal metabolites, such as zearalenone, found in foodstuff (e.g., grain) also belong to the phytoestrogens. Reproductive disorders in sheep are well documented after the eating of red clover containing high amounts of genistein (*259*). Some of the food products rich in phytoestrogens include rye, wheat, cabbage, sprouts, spinach, and soybean. Soybean is by far the richest source of plant estrogens and is used ubiquitously in the food industry as a protein source, including infant milk formula substitutes. Phytoestrogens may not bioaccumulate or biomagnify but they are readily metabolized and excreted (*260*).

Phytoestrogens have been shown to have estrogenic effects both *in vitro* and *in vivo* (*212,258,261*). Feeding of rams with clover that is rich in isoflavone resulted in decreased sperm counts (*262,263*). The effects are receptor mediated and depending on the dose are either estrogenic or antiestrogenic in an adult animal (*264,265*). Because of these potential antiestrogenic effects, high doses of isoflavones in the diet have been proposed as being beneficial for reducing the risk of hormone-dependent cancers (*266*). However, little consideration has been given to neonatal and childhood life when exposure to phytoestrogens would be presumed to have nonbeneficial effects.

Other Xenoestrogens

Bisphenol-A, a plastic monomer that was released from polycarbonate flasks during autoclaving, was shown to have an estrogenic effect on MCF-7 breast cancer cells (*267*). Notably, bisphenol-A is used extensively as a plasticizer, e.g., it is used in the lacquer coating of food cans that are then heated for sterilization purposes (*268*). Other common chemicals used in the plastic industry include the phthalate esters, butylbenzyl phthalate and di-*n*-butylphthalate, which were shown to act as weak estrogens on breast cancer cells (*203*). In the E-SCREEN assay, di-*n*-butylphthalate was not estrogenic (*204*). Neither of the phthalate esters had antagonist effects, suggesting that their action may be cumulative (*203*). Phthalates are the most abundant man-made environmental pollutants; and human intake per day by various routes, especially through the diet, is measured in tens of milligrams (*201*). A food antioxidant, butylated hydroxyanisole (BHA), was also estrogenic in breast cancer cell assays (*203*). However, the estrogenic potency of BHA and phthalate esters was lower than that of octylphenol or *o,p'*-DDT.

Summary

Numerous environmental chemicals, such as many organochlorine pesticides, PCBs, alkylphenol polyethoxylates, phthalates, and phytoestrogens are known to have estrogenic effects in vertebrates or in assays *in vitro*. However, only a few of the tens of thousands of man-made chemicals have been tested for estrogenic or other endocrine activity, and therefore, it is highly possible that other estrogenic chemicals remain unidentified. A major problem at present is how to fill this gap in our knowledge rapidly and cost effectively.

Exposure of Humans to Environmental Chemicals with Estrogenic Activity and Their Effects on Male Reproductive Health

The major routes of exposure to man-made chemicals are thought to be

- Dietary: pesticides, (including chemicals used in the formulation of commercial products), food additives (including synthetic flavoring substances), contaminants (such as PCBs, dioxins, metals, industrial chemicals, especially those that are biomagnified in food chains), packaging and wrapping materials (e.g., plastics, food wraps)
- Environmental: from the pollution of air and water
- Domestic: from contact with household products, cosmetics, clothing, and probably many others
- Occupational: inhalation, dermal contact and ingestion of active compounds, depending on the occupation.

Occupational exposure has not been considered in any detail in this report, which is aimed at describing the situation for the general population. However, valuable information on possible association between exposure to chemicals and effects on humans may originate from studies in occupational settings where high exposures have taken place. Because of better documentation and higher exposure, such studies are more likely to reveal adverse effects of chemicals on humans than are the studies on the general population (*269*).

The diet is usually regarded as the most important source of foreign chemicals. Very preliminary estimates of some exposures in Denmark are included in "Appendix A." It should not be overlooked that the exposure via routes other than food, such as air, drinking water, and particularly the skin (e.g., detergents) may be highly significant. Current knowledge on actual exposures is rather limited, in particular with regard to the domestic exposure to chemical products. A close examination of all possible exposures to chemicals suspected to be environmental hormones has not been possible within the constraints of this report. Humans may be exposed to xenoestrogens in multiple ways. Direct administration of synthetic hormones, such as DES or ethinyl estradiol is obvious, but the same hormones may be found in meat and dairy products in some countries (*270*). Cow's milk has a high concentration of endogenous estrogens during late

gestation, and this milk is also collected for human consumption. The ratio of estrogens in this milk to that in plasma is generally greater than one (*271*), and therefore much higher than in human breast milk. Occupational exposure in the pharmaceutical industry is a possibility for a small minority. Estrogens occur in measurable amounts in sewage effluent used for irrigation (*272*). Phytoestrogens are ingested in large amounts, and weakly estrogenic alkylphenolic compounds are applied to the skin and ingested daily. Important issues to consider are the quantity of estrogenic compounds present, their potency, their capability to bioaccumulate and biomagnify, and their additive, synergistic, or antagonistic effects. Concentrations of organochlorine contaminants in human reproductive tissue, adipose tissue, and blood from the general population (Table A1) and in human breast milk (Table A2) worldwide are included in "Appendix 1"[adapted from Thomas and Colburn (*206*)].

Organochlorine Pesticides and Polychlorinated Biphenyls

The daily intake of DDT is now small in Europe and North America, and it may not have a significant influence alone. Chlordecone (Kepone), which is estrogenic, caused an occupational risk to workers exposed to high levels of the compound: exposed men had oligozoospermia, decreased sperm motility and abnormal sperm morphology (*273*).

Exposure to several organochlorine pesticides and PCBs at the same time may lead to untoward effects, as exemplified by the suggested association between slight disturbances of development in children exposed *in utero* and contaminants present in Lake Michigan fish (*274–278*). The risk was related to lifetime intake of contaminated fish by the mothers and to PCB levels in maternal serum and milk rather than to intake during pregnancy. Reproductive disorders have not been described, but the affected male children have not yet reached reproductive age. These studies have been extensively debated, and more investigations in populations with well-known high exposures are needed to resolve this issue.

Levels of some PCB congeners were inversely correlated to sperm motility in semen samples in which sperm concentration was < 20 million/ml (*279*). The small number of subjects in the study leaves the significance of the finding open. Severe poisoning accidents occurred in Yusho, Japan, and Yu-Cheng, Taiwan, where a large population ate rice oil contaminated with PCBs, PCDFs, and polychlorinated terphenyls. Exposed pregnant women had increased fetal loss and the surviving infants had low birth weight (*280*). The exposed infants were delayed in their developmental milestones. The behavioral effects were probably mainly due to exposure *in utero*, since the effects were also found in bottle-fed infants (*232*). The boys at pubertal age (11 and 14 years of age; $n = 23$) exposed prenatally to very high levels of contaminants had significantly smaller penis lengths when compared to controls, whereas 31 boys at prepubertal age (8 and 10 years of age) did not differ from controls (*281*). The plasma levels of PCBs in Yusho were originally 0.01 to 0.1 ppm while body fat levels ranged up to 76 ppm. In the normal population the corresponding figures are < 0.005 ppm and 0.5 to 10 ppm (*282*). The reproductive ability of the exposed males has yet to be ascertained.

Dioxins and Furans. Humans are normally exposed to dioxin and furan levels far below those that induce reproductive disorders in animal experiments (*206*). After the Seveso accident in Italy in 1976 where a factory exploded during the production of 2,4,5-trichlorophenol, TCDD serum levels on a lipid basis ranged from 1770 to 56,660 ppt (ng/kg) in exposed children (*283*), while the background concentration in human milk fat is approximately 2 ppt (*225*). No reproductive disorders in adults have been described after the accident (*284,285*), though the exposed male children have not yet been evaluated.

Recently Found Xenoestrogens

Measurement of human exposure to alkylphenols, phthalate esters and bisphenol-A is difficult because the data are still sparse. There is, however, considerable concern because these compounds are so ubiquitous in the modern environment. Some nonylphenol ethoxylates and one octylphenol ethoxylate were found at concentrations of 15 to 29 ng/liter in drinking water in New Jersey (*286*). In different fish species, concentrations of 0.13 to 3.1 mg/kg dry weight of nonylphenol and its two ethoxylates have been found (*256*). Since these compounds have very weak estrogenicity, it is too early to estimate whether environmental exposure to them has any influence on humans, as the level of exposure of man is not known. However, for phthalates it is established that human intake per day is likely to be several hundreds of micrograms per kilogram per day, from certain food sources alone (*201*). This level of intake would be likely to result in estrogenic effects based on data from *in vitro* (*203*) and *in vivo* (*200*) studies, though not all phthalates may be estrogenic.

Phytoestrogens

Large amounts (hundreds of milligrams) of phytoestrogens such as isoflavones can be ingested daily by humans, especially in a vegetarian diet. Antiestrogenic action of isoflavones was demonstrated in women who consumed a soy protein-enriched diet containing 45 mg isoflavones daily for 1 month: the follicular phase was prolonged, and the midcycle surge of LH and FSH was suppressed (*266,287*). This may have occurred because of phytoestrogen-induced production of SHBG, which would reduce bioavailability of endogenously produced estrogens. In postmenopausal women, dietary soy induced no significant estrogenic or antiestrogenic effects (*288*). SHBG and gonadotropin levels remained unchanged in these women, whereas the vaginal epithelium showed a tendency for a higher percentage of superficial cells (indicative of estrogenicity). A study of postmenopausal women in Australia suggested an estrogenic influence by phytoestrogens on the vaginal epithelium (*289*). Many infants are fed with soy-based milk-substitute formulas rich in phytoestrogens. There are no data available about the possible endocrine effects in children, but it is presumed that the situation would be radically different from the adult because of the negligible endogenous production of estrogens in infancy (especially in male infants). Female rats exposed neonatally to phytoestrogens show an increased incidence of premature anovulatory syndrome in adult life. This syndrome is recognized as a classical consequence of inappropriate exposure to estrogens in neonatal life (*290*). Many phytoestrogens, such as lignans and isoflavonoids, are metabolized and excreted in urine similarly to endogenous estrogens (*260*). Thus, they may not bioaccumulate in the body.

Summary

Humans are exposed to environmental estrogens in multiple ways. Diet, drinking water, air, and the skin are the routes through which xenoestrogens enter the body. Several of the known xenoestrogens have a weak estrogenic activity but are highly persistent and accumulate in fat.

Moreover, many of them have additive effects. Some of these compounds, such as phthalates, are present in high concentrations in certain food items, whereas concentrations of pesticides and PCBs are very low. The level of exposure to pesticides and other contaminants is rather well monitored, whereas very little is known about exposure to other xenoestrogens.

Methods for Evaluation of Estrogenlike Effects, Human Exposure to Estrogenic Compounds, and Trends in Male Reproductive Health

The complexity of our chemical environment is increasing continuously, and it may not be possible to forecast all of the effects of compounds released in nature. However, we have to use all the available methods and develop new ones to monitor possible adverse effects of chemicals and natural compounds on human and wildlife. Reproduction is a major concern because disturbances of this process rapidly threaten populations as a whole. The male reproductive system is very sensitive to the influence of an excess of estrogen; therefore, estrogenlike effects in the environment are a primary suspect for causing the increased reproductive disorders of men and wildlife animals.

Test methods are needed to screen chemicals for estrogenic effects. Further methods are needed to assess estrogenic exposure of humans and other species. Methods to analyze the mechanism of action of estrogenic compounds are necessary. And finally, it is important to assess the reproductive toxicity of chemicals and to predict their effects in the environment, including the effects on organisms, populations, communities, and ecosystems.

Epidemiology

The general research question is whether exposure to environmental estrogens, in particular during fetal or neonatal life, has adverse effects on the male reproductive system. When designing epidemiological studies to evaluate this question, one has to consider both exposure and reproductive health outcome. Exposure measurements may sometimes be directly available, e.g., controlled DES treatment during pregnancy, but in most cases such information has to be obtained retrospectively. This, however, may raise difficulties of recall bias and recall uncertainty (often an answer to a general question such as "have you had hormone treatment during pregnancy" will be the only exposure data that can be obtained).

In this situation the so-called ecological approach may be necessary. Rather than relating adverse outcomes to exposure for individuals, this is done for groups. Two general dimensions are used: temporal and spatial. Indeed the temporal trends in selected outcomes have been instrumental in raising the suspicions and concerns described in this report. It is hypothesized that putative exposures do vary considerably along the temporal gradients. Spatial trends have so far been exploited in only a few cases, e.g., Danish–Finnish sperm concentration and testicular cancer incidence (*16,21,22*), which also illustrates that covariation of outcomes can sometimes be informative when few if any exposure measurements are available. In general we are not aware of much hard evidence to directly support an explanation in terms of specific exposures of the spatial gradients. Sources of variation in the spatial gradient are, for example, ethnic differences, with the associated difficulties in separating genetic, cultural, and socioeconomic gradients from the exposures of our prime interest. In particular, the considerable success from nutritional epidemiology of migrant studies (such as Japanese immigrants to Hawaii or California) might form an inspiration for epidemiological designs for the problem of environmental estrogens.

Turning to outcomes, the original focus was on defects of the male reproductive system. To this we have added specific estrogen responses (gynecomastia, male breast cancer). Schematically, there are advantages and problems (Table 2).

Responses occurring with delay require a long time span for these kinds of reproductive studies. For both prospective and retrospective studies this has negative consequences for both logistics and interpretation (e.g., male breast cancer cases may have to be related to pregnancies 50–70 years ago).

Epidemiological studies are needed to monitor the trends in incidence of testicular cancer and birth defects (cryptorchidism, hypospadias), and changes in semen quality and infertility. Follow-up of semen quality is very important, since the sperm concentration has decreased drastically during the last two generations (*5*) and the declining trend appears to be continuing (*11*). Risk factors for testicular cancer have been analyzed in several case–control studies. Estrogen treatment of mothers whose sons have developed testicular cancer has remained an equivocal risk factor. It is unfortunate that there are no prospective studies yet concerning the testicular cancer risk among males exposed to DES. The studies by Bibbo and Gill, and their co-workers (*174,291*) preceded the time when testicular cancer would have become manifest. Epidemiological studies of the risk factors for poor semen quality and infertility are needed. Furthermore, epidemiological studies should be combined with studies on hormone metabolism when a link between disorder and hormone effect is sought. In women, dietary influence on estrogen metabolism and the risk of breast cancer was analyzed in large epidemiological studies in which both exogenous and endogenous hormones and their metabolites were analyzed meticulously in blood, urine, and feces (*292*); this provided the basis for the link between high levels of free endogenous estrogen (found in women with Western-type diet) and increased breast cancer risk. There are no comparable studies among men concerning reproductive disorders, although if these disorders are induced in fetal/childhood life

Table 2. Assessment of outcomes.

Outcome	Advantages	Problems
Hypospadias	Inborn: fast ascertainment Available in some registries	Not uniformly recorded; no generally agreed diagnostic criteria
Cryptorchidism	Inborn: fast ascertainment Available in some registries	Spontaneous descent; no generally agreed diagnostic criteria
Semen quality	Widely available and reasonably uniform sperm concentration measurements; sperm output probably related to the Sertoli cell number	Functional importance of sperm concentration unknown, other parameters not often uniformly measured
Fecundity	Direct importance	Essentially impossible to measure separately from confounding factors
Testicular cancer	Well registered, important	Rather rare
Male breast cancer	Well registered, important	Very rare, occurs late in life

but are not manifest until adulthood, then there are large obstacles to deducing any cause-and-effect relationships. "Time to pregnancy" analysis and exposure assessment allow the study of fecundity of exposed males (*293*).

Ecoepidemiology

Causal relationships between environmental contaminants and specific disease states in wildlife are difficult to obtain because these animals are not living in controlled conditions. An ecoepidemiologal approach to circumvent these problems is to evaluate systematically the relationship between proposed causal agents and specific outcomes. The criteria used to develop causal inference include *a*) probability, *b*) time order, *c*) strength of association, *d*) specificity, *e*) consistency on replication, *f*) predictive performance, and *g*) coherence [reviewed by Fox (*294*)]. This approach is often more useful in rejecting a causal relationship than in providing definitive support. However, the use of this systematic approach, well-formed sampling designs, and rigorous statistical analysis can provide us with data useful in risk assessment.

Continuous monitoring of the wildlife populations is needed to detect any disturbances in reproduction. Furthermore, timing and magnitude of chemical exposure should be considered when estrogenic effects are examined.

Experimental Models for Testing Estrogenicity and Estrogenic Molecules

Different approaches can be used to assay estrogenic activity, depending on whether or not the compound is known to have such an activity. In the first case, direct physicochemical analyses, such as gas chromatography–mass spectroscopy or high-performance liquid chromatography (HPLC), can be used to assess the presence, and if necessary, the concentration of a xenoestrogen(s). Many banned chemicals (e.g., PCBs) can be detected by this method. Specific immunoassays can be used when appropriate antibodies are available. Different approaches must be used when putative estrogenicity of a compound(s) is screened or the cause of a suspected estrogenic contamination is searched for. Binding assays and bioassays are commonly used for this purpose.

The principle of binding assays is to try to displace radiolabeled estradiol from its receptor by increasing molar concentrations of either unlabeled estradiol (reference curve) or different purified xenobiotics to be screened. Biological material used here is either a total extract or a nuclear extract of cells or a tissue containing a high concentration of estrogen receptor (e.g., the rabbit or rat uterus, MCF-7 cells). Binding experiments are very useful, but it should be remembered that binding of a compound does not necessarily imply its biologic activity. Moreover, the concentration of a chemical needed to displace 50% bound [^{3}H]estradiol is orders of magnitude higher than that needed to elicit a biological response.

There are a number of bioassays available for assessment of the estrogenicity of a chemical. These can be divided into two groups: animal models and *in vitro* models.

Animal Models. A range of different species such as chicken, rat, mouse, trout, and reptiles has been used in estrogenicity testing. The most widely used rodent bioassay measures the increase in uterine weight in the rat (*295*). However, it is well known that this bioassay gives a crude estimate of an estrogen effect, that it is insensitive, costly, laborious, and cannot be adopted for large-scale screening. Moreover, there is no standard procedure for the uterotropic assays. Instead, different laboratories use different protocols (multiple doses vs single dose, 24 vs 72 hr, etc.). Vitellogenesis in male fish, e.g., trout, can be used to test for the presence of estrogenic compounds in the aquatic environment.

Mammalian animal models. Various laboratory or domestic animal species can be used to address specific aspects of the possible effects *in vivo* of estrogenic chemicals. These should be particularly useful for studies of delayed effects (i.e., where exposure to the chemical(s)occurs in fetal/neonatal life and the reproductive consequences are manifest in adult life) and for studies of the effects of chronic low-level exposure to xenoestrogens. For example, possible effects of these chemicals on Sertoli cell multiplication can be studied in developing rats or mice by exposing them during gestation and/or during the first 3 postnatal weeks of life (the period of Sertoli cell multiplication extends from day 16 of gestation to postnatal days 15–18 in the rat). Sperm output, fertility, and testis size and morphology can then be evaluated in adult life and Sertoli cell number determined in other animals at around day 18 of postnatal life. In this way, possible chronic effects of low-level exposure to xenoestrogens during development on adult reproductive ability can be evaluated in a fairly simple way. Such studies are ongoing and have already proved that such effects can be detected (*200*). The importance of these particular studies is that they may provide reference values of harmful exposure (i.e., µg/kg/day) that can then be related to measured levels of exposure in man.

The other main uses of mammalian animal models will be in determining the mechanisms of xenoestrogen-induced disruption of reproductive development. Mice and rats have been used already for some such studies, and probes or antibodies for some of the key genes that are expressed during normal reproductive development are available. Evaluation of the effects of exposure to xenoestrogens at specific times during development on expression of these key genes (or on the effects of the gene products) will obviously be extremely valuable, especially as any changes identified will be able to be related to consequences in adult life.

There is also a need for exploitation of animal models other than rodents, as these have limitations in terms of direct access to the developing male fetus (i.e., exposure is always through the mother). In this respect there is a place for the use of the fetal sheep or pig and the developing opossum. In the former, it is possible to cannulate blood vessels in the fetus at mid-term and to then directly administer a compound to the fetus and to monitor consequences in terms of changes in blood hormone levels (*165*); for example, this system could be used to determine what blood level of xenoestrogen in the fetus results in reduced secretion of FSH from the pituitary gland and is thus likely to reduce Sertoli cell multiplication (see "Effects of Synthetic Estrogens on the Testis in Animal Models"). In contrast, the opossum provides fairly unique direct access to a fetus that is born before sexual differentiation has occurred. Thus administration of xenoestrogens to the young at this stage (either through the skin or orally) could provide a direct and controlled way of pinpointing when and how such exposure might result in disruption of the normal cascade of male reproductive development; this would enable identification of critical windows of developmental susceptibility to estrogens. It is established already that *a*) the pathway of development in these animals is comparable to that in eutherian mammals, and *b*) exposure of the young to estrogens is able to disrupt completely the normal differentiation of the testis and seminiferous cord formation (*296*).

Vitellogenesis. Vitellogenin is a yolk protein produced normally in the liver of

female fish under estrogen control (*297*). Very little, if any, vitellogenin can be detected in male fish (*298*). However, exposure to estrogens activates the vitellogenin gene, resulting in increased vitellogenin levels in the blood of male fish, and clear dose–response effects have been documented (*299*). Vitellogenesis can be used both in field studies and in laboratory *in vitro* studies (*300*). It is measured by a specific radioimmunoassay (*301*). Sex reversal could also be used as an end point for estrogenic effects in an aquatic milieu.

In field studies, caged male rainbow trout are exposed to water in areas of interest, and their vitellogenin levels are followed over time (*112*). This is a good screening method for the presence of estrogenic compounds in different aquatic habitats. Vitellogenin production by superfused trout hepatocytes can also be used as a sensitive *in vitro* bioassay for analysis of estrogenic compounds in the water (*111*).

Fish reproductive tests. The Office of Pollution Prevention and Toxics (OPPT) in the U.S. Environmental Protection Agency (U.S. EPA) is using two fish reproductive assays: *a*) fish partial chronic toxicity test that measures effects from adult to the early life stage, and *b*) fish whole life toxicity test that measures effects from egg to early life stage to adult and then to early life stage (OPPTS 850.1500).

Reptile egg assays. Reptile sex differentiation is temperature- and estrogen-dependent (*155*). Exposure of turtle eggs to a putative xenoestrogen by spotting or painting them with the compound results in a high proportion of feminization or intersex conditions of hatchlings in a temperature that would normally result in 100% males (*99*). This assay is suitable for studies of estrogen exposure in wildlife.

In Vitro *Assays.* Two complementary aspects can be studied by *in vitro* assays: proliferation of an estrogen-dependent cell line, and the induction of an estrogen-controlled function. In the first case, cell number and the incorporation of radiolabeled thymidine are the parameters measured. In the second case (induction of function), there are several possibilities. Prolactin production by pituitary cells in response to an estrogenic compound was an early attempt to measure estrogenic activity *in vitro*, but the assay was abandoned because of its nonspecificity. For assessment of the estrogenic activity in the aquatic environment, the *in vitro* assay for detecting vitellogenin in fish is extremely useful (*302*). At present, the most commonly used mammalian *in vitro* assays are the MCF-7 cell tests and the recombinant yeast cell assays. Serum that is required in the culture of breast-cancer cell lines must be charcoal-stripped to remove endogenous estrogens.

MCF-7 cell line. Human breast cancer cell lines (MCF-7 cells) that are sensitive to estrogen have been used to screen chemicals for their estrogenic effects (*81*). The test — also called the E-SCREEN — is based on the dose–response relationship between the proliferation of MCF-7 cells and the amount of estrogen to which the cells are exposed during 6 days of culture. Estradiol is used as a standard. By comparing the effects of a xenoestrogen and estradiol, we can present the relative estrogenic potency of a compound. Soto et al. (*81*) use the following concepts to describe estrogenicity: "Concentration" denotes the dose at which an estrogenic effect is detected; proliferative efficiency (PE) measures the ratio between the highest cell number in the presence and in the absence of estrogen; relative proliferative efficiency (RPE) measures the ratio between the maximal cell yield achieved by a xenobiotic and that of estradiol; relative proliferative potency (RPP) measures the ratio between the dose of xenobiotic and that of estradiol needed to achieve a proliferative effect. Alkylphenols, pesticides, phytoestrogens, and synthetic estrogens have been analyzed using this test (*81,212*). Some endocrinologists consider cell proliferation a hallmark of estrogen action; and if a compound does not have this property, regardless of what other end points it influences, it should not be called an estrogen. The E-SCREEN is technically an easy method and can be used for screening and assessment of approximate estrogenic potency of a compound. Other hormones and growth factors tested so far have not influenced this assay (*204*). However, the possibility remains that some compounds may influence these cells through other than estrogenic pathways and thereby confound the results.

MCF-7 cells were also used to detect the estrogenic effect of bisphenol-A that was released from polycarbonate flasks during autoclaving (*267*). Progesterone receptor induction and [^{3}H]thymidine incorporation combined with DNA measurement were used to measure estrogen activity (*81*). This approach covers the proliferative effect (thymidine incorporation) and the change-of-function effect (progesterone receptor induction) of the compound studied. This adds to the technical difficulty and the expense of the assay.

Estrogen effects on MCF-7 cells can also be monitored after transient transfection of cells with reporter plasmids pTKLUC and pERE-TKLUC by assaying for luciferase activity (*203*). The reporter plasmid pTKLUC contains the herpes simplex virus thymidine kinase (TK) promoter inserted in the Bgl II site of the luciferase reporter plasmid pGL2-Basic (Promega). pERE-TKLUC contains a single copy of the vitellogenin A2 estrogen response element inserted upstream of the TK promoter in pTKLUC.

ZR-75 cell line. Several human estrogen-responsive cell lines, such as ZR-75, in addition to MCF-7 can be used in cell proliferation assays (*203*).

Recombinant yeast cell lines. The most sensitive estrogen measurement at the moment is a recombinant cell bioassay comprising a yeast cell line (*Saccharomyces cerevisiae*) transformed with plasmids encoding the human estrogen receptor and an estrogen-responsive promoter fused to the structural gene for β-galactosidase (*303*). This assay detects 100-fold lower estradiol levels than the widely used traditional radioimmunoassays. However, the sensitivity of the assay to other estrogenic hormones than estradiol is lower. This bioassay revealed an 8-fold difference in the estrogen levels of prepubertal boys and girls (*303*), who were previously believed to have similar hormone concentrations (*304*). This has important implications for future studies since it is now possible to measure quantitatively increased estrogen levels in prepubertal boys. The assay is simple to perform once the cell line is established since only the measurement of β-galactosidase activity is needed to observe the effect.

Sensitivity of the Assays. The sensitivity of the assays for measurement of 17β-estradiol is given in the Table 3.

The recombinant yeast cell assay appears to be the most sensitive of the assays, although the E-SCREEN may not be far behind. Both assays detect estradiol levels that are less than 0.1 pg/ml. Traditional radioimmunoassays with an antibody strictly specific for estradiol had a maximum sensitivity of 2 pg/ml (*305*). However, sensitivity can be improved to less than 0.1 pg/ml (*306*). In the E-SCREEN assay, the lowest estradiol level to produce a maximal proliferative effect is 10 pg/ml, but detectable proliferation occurs already at 0.3 pg/ml (*307*). Activity, significantly different from the unstimulated control, has been detected at 0.03 pg/ml (C Sonnenschein and AM Soto, personal

Table 3. Sensitivities of estrogen assays.

Assay	Sensitivity	References
Radioimmunoassay (RIA)	Traditional RIA: 2 pg/ml Improved RIA: 0.3 pg/ml	Korenman et al. (*305*) Legan et al. (*306*)
MCF-7 cell assay (E-SCREEN)	0.03–0.3 pg/ml	Soto and Sonnenschein (*307*) AM Soto and C Sonnenschein, personal communication
Recombinant yeast cell assay	0.02–0.2 pg/ml	Klein et al. (*303*); JP Sumpter, personal communication

communication). When the ultrasensitive estrogen assays are used, extreme care should be taken in the way the samples (to be analyzed) are handled, stored (quality of plastic vials), and processed, because major problems may be encountered from low levels of contaminating estrogens, e.g., plasticizers leaching from plastic storage tubes, syringes, cell culture plates, etc.

Complementary to these techniques, the binding of the compounds to proteins, such as SHBG and α-fetoprotein (AFP), should always be studied in order to address the difficult problem of bioavailability.

In conclusion, although a wide variety of different assays are used (and probably always will be!), in general they have produced very consistent results; that is, a chemical that is estrogenic in one assay is estrogenic in all other assays. For example, alkylphenolic compounds are weakly estrogenic in molecular assays (*257*), cell proliferation assays (*81*), and *in vitro* estrogen-binding assays (*203*). There appears to be no species specificity; i.e., a chemical that is estrogenic in one animal appears to be estrogenic in all other species. Again, alkylphenolic compounds provide a good example. These chemicals are weakly estrogenic in fish, birds, and mammals (*257*). This is not at all surprising since the structure of the estrogen receptor is highly conserved between species.

Limitations of* in Vitro *Testing. Absorption and metabolism of a compound are important for its effects. Because a chemical is, or is not, estrogenic *in vitro* does not necessarily mean that it will exhibit this activity *in vivo*. It may not be absorbed. It may be metabolized in the gut to an inactive or nonabsorbable metabolite. Alternatively, a chemical that is nonestrogenic *in vitro* may be converted to an estrogenic metabolite in either the gut or the liver. This possibility is important as it points out the limitation of *in vitro* screens (false negatives). Screening of classes of compounds and identification of the chemical structure(s) associated with estrogenicity are obvious ways by which this limitation could be minimized.

Although some proestrogens are supposed to be metabolized into an active form in the liver, and thus they are expected to be inactive in *in vitro* assays, such compounds (e.g., alkylphenol monoethoxylates, some PCB congeners, methoxychlor) have been found to be estrogenic for MCF-7 cells, suggesting that these cells may also metabolize some chemicals (*204,257*).

General Reproductive Toxicity Testing

Only a few chemicals have been tested specifically for estrogenic or other endocrine activity. However, many chemicals have been tested for their reproductive toxicity, and thereby indirectly for estrogenicity, although weak estrogenic activity most probably does not appear in these tests. Current toxicity tests are designed primarily to detect overt teratogenic and reproductive effects or toxicity in the adult—they have not been designed to seek out effects where there is a long lag-phase between induction and manifestation of the effect. Moreover, unless such changes were gross and pathological, they would probably be dismissed as insignificant, e.g., small reduction in testis size and sperm count.

Toxicity testing used by U.S. regulatory agencies and by the National Toxicology Program includes three test segments (Segments I–III), Fertility Assessment by Continuous Breeding (FACB), and either two-generation or multigeneration tests (*308*). Segment I covers fertility and reproductive function in male and female; Segment II deals with developmental toxicology and teratology; and Segment III tests perinatal and postnatal toxicity.

Thus, reproductive toxicity testing belongs to the routine toxicology that has to be undertaken before a new compound can be released to the environment. The problem is that although testing appears extensive, it may miss important late effects and actions that occur only when additive factors are present. The reproductive toxicity tests used in the current safety assessment of various chemicals are discussed in more detail in "Appendix B".

Testicular Cancer Models

There are no animal models for testicular germ cell tumors. This necessitates more intensive search for such a model. Testicular stromal tumors can be produced in mice both by gene deletion (*309*) and by overexpression of an oncogene under the control of a testis-specific promoter (e.g., inhibin alpha promoter connected to SV 40 large-T antigen). The transgene approach has been used to produce testicular somatic cell tumors from which new testicular cell lines have been developed (K Kananen, personal communication). Direct transformation of germ cells with SV 40 large-T antigen has been used to immortalize spermatogonia (*310*). When a temperature-sensitive p53 mutant is co-transfected into these cells they were reported to differentiate through meiosis (*311*). These recombinant cell models may give possibilities for detailed analyses of many features of gene activity and its regulation in specific testicular cell types. Although the cells are transformed, most of their characteristic functions should remain unchanged. Such approaches may be extremely useful in improving our understanding of primordial germ cell development and of the factors that are involved in arrest of development resulting in precancerous carcinoma *in situ* (CIS) cells.

Brinster and co-workers (*312,313*) demonstrated that stem cell spermatogonia survived a transit to mouse seminiferous tubules that were either partially or totally missing their own germ cells. The transferred spermatogonia produced normal spermatozoa. Spermatogenic cells survive poorly *in vitro*, but spermatogonia are the most viable (*314–316*). This raises the possibility of *in vitro* experimentation, which can be continued *in vivo* by injecting cultured spermatogonia back into the seminiferous tubules. Human CIS cells could be transferred to mouse seminiferous tubules (which are an immunologically privileged site) to follow possible carcinogenesis. Endocrine manipulation of recipient animals would enable study of the role of hormones in the promotion of tumor growth.

Summary

Multidisciplinary approaches are needed into studies of environmental effects on male reproductive health. Epidemiological methods should be used to follow up the trends in male reproductive health and to identify putative association to environmental factors. Similarly, ecoepidemiology gives valuable information on concurrent

changes in wildlife. Experimental studies in laboratory animals and cell lines provide direct evidence of the effects of xenobiotics. Both physicochemical and biological methods should be used for the identification of estrogenic-/endocrine-disrupting compounds in the environment and for the determination of human exposure to these agents.

General Discussion on the Association between Chemicals in the Environment and Male Reproductive Health

The possibility that exposure of humans and wildlife to environmental estrogenic chemicals might result in adverse changes in reproductive development, function and/or behavior is not particularly new; concern was first expressed two decades ago in relation to DDT. Indeed, the first demonstration that a range of man-made chemicals could be estrogenic when administered to animals stems from 1938 (*317*). The issue has resurfaced because of two new developments. The first is the increasing appearance in the human population of several adverse changes of the sort that researchers predicted might occur if human exposure to environmental estrogens was widespread. The second has been the discovery of many new environmental estrogens to which we are exposed daily. In light of these developments, the foregoing report has reevaluated the strength of the available information to ascertain whether or not there is real concern for human health stemming from exposure to environmental estrogens, or whether the data are more consistent with a theoretical, but unlikely, effect on the human. The following stepwise conclusions have been reached:

- All of the best evidence available points with some certainty to a rising tide in Europe and many other countries of human male reproductive disorders involving sperm counts (and probably sperm quality), testicular cancer, malformation of the external genitalia, and possibly testicular maldescent.
- There are insufficient data to prove or disprove that these adverse changes in male reproductive health are the result, wholly or partially, of exposure to environmental estrogens. In addressing this issue, we have identified alarming gaps in our knowledge of the route and levels of human exposure to nonpesticide environmental chemicals. Many of these chemicals have not been evaluated for their reproductive toxicity; even where such studies have been performed, their design and intent were such that they may have failed to exclude delayed effects of the sort that can be induced by estrogen (or other hormone) exposure during development.
- If the above changes in male reproductive health do result from fetal and/or neonatal exposure to environmental estrogens, then we will not know what the current prevalence in male children is for another 20 to 40 years. This is due to the delay between induction and manifestation of many of the effects, e.g., lowered sperm counts or the development of testicular cancer in adult life. The indications are that these problems are still increasing in incidence in the general population.
- Based on what is known about the widespread imprinting effects of steroid hormones (androgens and estrogens as well as antagonists of these hormones) when there is exposure during fetal/neonatal development, it is possible that there are other adverse health consequences in man of such exposures of which we are currently unaware, e.g., permanent changes to the immune system, growth, etc.
- There is enough evidence from a variety of sources to suggest that environmental estrogens have the capacity to induce adverse minor and major reproductive defects in man, but there are inadequate data on the level of actual human exposure to these chemicals, which means that no accurate risk assessment can be made. There are also insufficient animal data to permit such an assessment.
- Determination of whether environmental estrogens do exert harmful effects in man will probably have to rely on weight of evidence rather than establishment of precise cause and effect. To provide this evidence as accurately as possible, future work should address the problem at several levels, including epidemiology, laboratory animal studies, wildlife studies, more complete identification of estrogenic chemicals (i.e., screening), and accurate assessment of the routes and levels of human exposure to these chemicals.
- There are substantial differences in the incidence of the reproductive defects, referred to previously, in different countries/races and these also appear to show possible relationships to the incidence of other hormone-dependent diseases of the reproductive system (breast, prostate). The etiology of these differences should be explored to ascertain to what extent they reflect ethnic/genetic, lifestyle, or environmental influences.
- There is an urgent need for a clearer understanding of the hormonal environment of the fetus during the period of male sexual differentiation and development, especially of what mechanisms are present to protect the fetus from maternal estrogens, e.g., α-fetoprotein, steroid-metabolizing enzymes, etc. Because of the inaccessibility of the human fetus at this stage, these studies will probably have to be undertaken in animals.
- Investment of resources in obtaining the information necessary to assess whether environmental estrogens pose a health risk to man will have major spin-off in terms of our understanding of fetal and neonatal determinants of disease, the routes and importance of human exposure to nonpesticide environmental chemicals and of the etiology of geographic and ethnic differences in disease.

Suggestion for a Strategy to Strengthen the Evaluation of Hazards of Estrogens or Xenoestrogens

Several lines of evidence have indicated adverse trends in male reproductive health over the last few decades. Clinical and experimental research has demonstrated that the reproductive disorders reported may be interrelated and may have a common origin in fetal life or childhood. Studies of wildlife and human epidemiology suggest that environmental factors are involved in the origin of reproductive disorders. Common environmental contaminants such as alkylphenols and phthalate esters, and natural factors such as phytoestrogens, have been shown to be endocrine-disrupting agents, many of them being estrogenic. Our hypothesis is that the adverse trends in human male reproductive health is, at least in part, associated with exposure to environmental estrogenic compounds during early development. Testing of this hypothesis necessitates a multidisciplinary research approach, including epidemiological and experimental studies, examination of wildlife, and analyses of environmental contaminants and human exposure to them. Extensive international collaboration that combines the existing strengths of various institutions and investigators is probably required if this complex

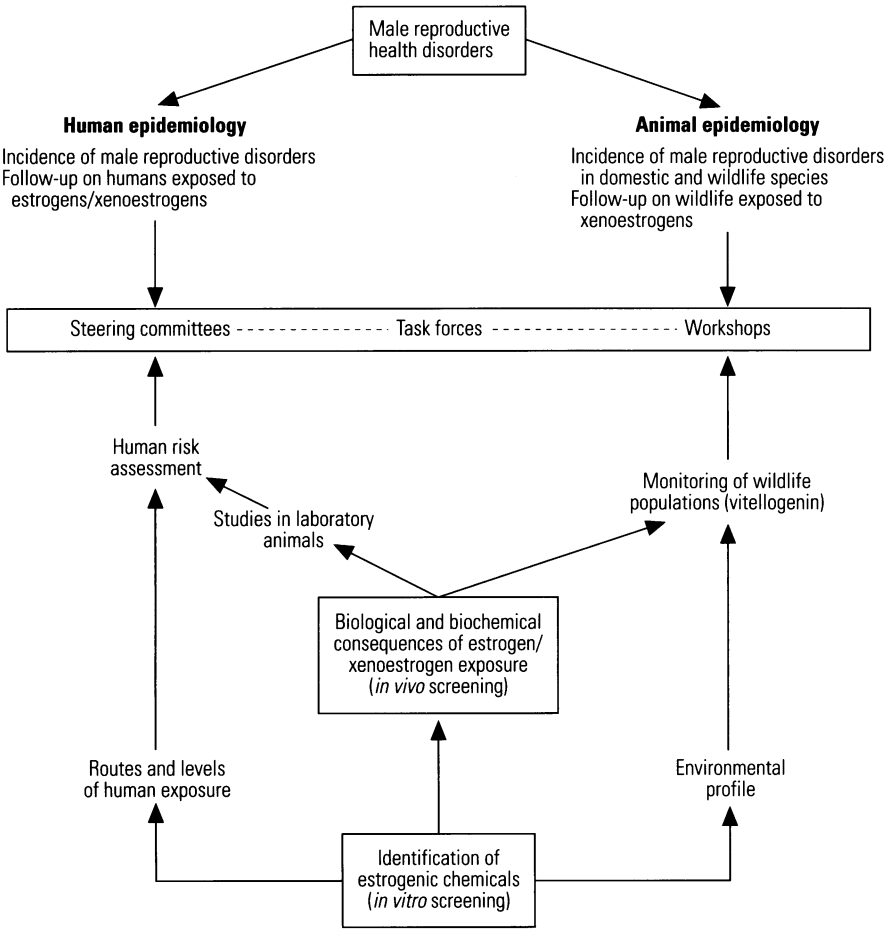


Figure 8. Suggestion for a research strategy to strenghten the evaluation of hazards of estrogens or xenoestrogens.

health problem is to be addressed in the most accurate and effective way.

Research Strategy

The proposed research strategy is illustrated schematically in Figure 8 and is expanded upon in the relevant sections below. There are three fundamental components to this strategy (boxed in Figure 8).

The first component centers on the collection and evaluation of epidemiological evidence for male reproductive disorders in man and animals. The intention of this approach is to define the scale of male reproductive disorders, to establish geographical differences in their distribution and to assess whether there is an increase in incidence of such disorders in groups with known exposure to estrogens or xenoestrogens.

The second and third components of the strategy are designed to identify xenoestrogens using a variety of *in vitro* screening methods and then to evaluate whether their administration to laboratory animals results in biochemical and biological effects (*in vivo* screening). This approach would be complemented by studies on the environmental distribution of these chemicals and by assessment of the routes and level of human exposure. This information, in conjunction with the relevant epidemiological evidence, should enable some form of risk assessment to be made for man and for wildlife.

Epidemiological Studies in Man

In epidemiological studies, trends in male reproductive health must be followed up further in different geographic areas and in populations with different ethnic and racial backgrounds. In addition, every attempt should be made to use some of the existing data or cohort studies, if these are appropriate, although in some instances collection of new data is essential.

Incidence of Male Reproductive Disorders in Man. There are geographic and racial variation in semen quality and other aspects of male reproductive function. Semen quality, testis size, levels of sex hormones and gonadotropins as well as presence of cryptorchidism and gynecomastia may all be indicators of abnormal male reproductive function. The aim of the study is to examine geographic and racial differences and to establish reference values for semen quality, blood levels of sex hormones and gonadotropins, testis size, and prevalence of gynecomastia and cryptorchidism, since these conditions may provide us with information on environmental and genetic influences on male reproductive development and function. Furthermore, such studies may be starting points for future prospective studies on secular trends in male reproductive function.

In order to obtain comparable groups of individuals, well-defined types of men should be investigated, such as army recruits and semen donor candidates. The study should involve men from the United States, France, Finland, Denmark, United Kingdom, and possibly Taiwan or Japan, with the final selection of countries to be determined by the study groups.

The following parameters should be assessed:

- semen quality: volume, density, morphology, computerized motility assessment; to ensure a standardization of the methods, the laboratories involved in the project should circulate relevant samples or videotapes
- serum levels of testosterone, estradiol, FSH, and LH
- morphometric measurements: weight, height, testis size
- cryptorchidism
- gynecomastia
- time to pregnancy if the men have reached an age when they have wished to become fathers

The rest of serum and semen samples should be stored in the freezer for possible future analysis for, e.g., environmental pollutants or other relevant parameters.

Denmark and Finland provide an interesting comparison. The incidence of testicular cancer in Denmark is remarkably higher than in Finland (*22*). Conversely, semen quality seems to be markedly better in Finland compared to Denmark (*16*). The incidence of testicular cancer is increasing in both countries, whereas less is known about semen quality. The incidence of cryptorchidism in Denmark in 1960 is known from a careful study of 2701 newborns (*60*). Additional information is available from the doctors' and midwives'

notes of 17,767 newborn males during the period 1957 to 1960. No information on possible increase in the incidence of cryptorchidism in Denmark is available and neither is there information on the incidence of other minor malformations of the male external genitalia. There are no large studies on the incidence of cryptorchidism in Finland. According to the hypothesis that testicular cancer, poor semen quality and developmental anomalies in the male reproductive tract are interrelated, we postulate that the incidence of cryptorchidism is higher in Denmark than in Finland and that the prevalence of genital malformations is increasing in newborns.

Follow-up Data on Humans Exposed to Estrogens/Xenoestrogens. There are situations in which exposure of pregnant women to estrogens or estrogenic compounds have been clearly identified. DES treatment in the United States and some countries of Europe or contamination of foodstuffs with PCBs and PCDFs in Japan and Taiwan are such examples.

These situations are true experimental models and maximum effort should be made to collect existing data on the offspring of these women. The following are the possibilities:

- Obtain additional information from men born to mothers who participated in the well-designed DES trial made in the United States in the 1950s (*167*). There have been more than 300 men in the follow-up studies of these cohorts (*174*).
- Obtain information from other men born to exposed mothers.

A possibility would be to study brothers of women known to be exposed to DES, as there is probably an increased possibility that they also were exposed to DES. The size of the study group and a paired control group would have to be defined carefully.

- New case–control studies should be done especially for testicular cancer, since, e.g., the DES-exposed males have now reached the age at which the incidence of testicular cancer peaks.
- Taiwanese boys prenatally exposed to PCBs are now in puberty (*281*). It is proposed to obtain all the available data on the reproductive tract disorders from these men, and later semen analyses when available.

More information is needed on the incidence and trends for genital defects in various countries.

- Attention should be mainly accorded to cryptorchidism, hypospadias, and male breast cancer. This information could be obtained from many, if not all, of the countries in the EU.
- Information from the newborn cohort established in the 1960s in Denmark could also be used to analyze the trends for genital malformations through a new study. The Bristol-coordinated European cohort study which involves large numbers of children in several Western and Eastern European countries will give additional data on these trends.

Risk factors may be shared among many disorders. Mothers of men with testicular cancer were reported to have an increased risk for breast cancer (*190*), suggesting common risk factors, such as a high estrogen exposure. In national cancer registries, it is possible to analyze the association between testicular cancer and mother's breast cancer in large patient groups, and such a study is ongoing for example in Denmark.

Occupational Studies. Studies on the offspring of women occupationally exposed to xenoestrogens. Comparison between population groups in so-called ecological studies may suffer from various weaknesses, including confounding bias. Also, if the main effect on male reproductive function is due to prenatal exposure, it may be very difficult to determine the causative exposure in any detail because it happened in the distant past. These problems do not indicate that such studies should not be carried out; but it would be important to supplement this evidence with studies of more specific exposures, such as those that occur in certain occupations.

Among the industrial chemicals so far identified as xenoestrogens are certain additives to plastic materials (e.g., alkylphenols, phthalates, bisphenol-A) and some pesticides. It would therefore be of interest to find female occupational groups with known exposures to such compounds. One group of particular interest is greenhouse workers. Because of the enclosed space of greenhouses, pesticide exposure invariably occurs, partly because of percutaneous absorption. Prospective and cross-sectional studies of the male offspring of the female greenhouse workers could then be carried out with emphasis on cryptorchidism and other indicators of adverse effects on reproductive organs.

Epidemiological Studies in Domestic Animals

There is a lack of information on possible secular trends in male reproductive function among domestic animals. Such animals might serve as good models for evaluation of the environmental impact on human male reproduction, although it should be kept in mind that, for breeding of many of these species, individuals with the best reproductive function have been highly selected. Nevertheless, if any adverse trends are still evident, this would provide powerful supporting data for the human studies and might open up new possibilities for identifying causal agents.

The species that might be of interest are the bull, pig, stallion, and dog. The indicators of male reproductive studies are, as in humans, cryptorchidism, hypospadias ,and semen quality. It is well known that dogs develop spermatocytic seminoma and not the classical, premeiotic germ cell-derived seminomas and nonseminomas. It remains to be seen whether such tumors occur among the other species. Available retrospective data (e.g., databases for artificial insemination stations) on reproductive function in these animals should be analyzed. New, prospective studies should be initiated, if suitable studies can be designed and the archival data warrant such investigation. The overall importance of these analyses is that they may indicate whether adverse changes in male reproductive function are unique to man or more prevalent in man.

Wildlife Studies

If environmental estrogens are currently present in biologically significant background concentrations, a survey of wildlife would provide important supportive information concerning possible human risk. One extremely useful biomarker of estrogen exposure is the synthesis of the hepatic protein vitellogenin. This is normally synthesized in the liver of egg-laying females after estrogen stimulation. Synthesis of this protein is usually correlated with seasonal reproductive activity in females but it is not found in males. Numerous laboratory studies, with a variety of wildlife species, have demonstrated that vitellogenin synthesis is stimulated in males after exogenous estrogen treatment. Recent evidence (*112*) suggests that both caged male trout in English rivers receiving sewage effluent and males of native fish species from various English rivers and from the river Seine in Paris exhibit elevated plasma vitellogenin levels. These data suggest that environmental estrogens exist in these rivers at biologically significant levels. Thus, a survey of male fish, amphibians, reptiles, and birds living in wetland areas would provide a

definitive measure of estrogen exposure in this environment.

Fish would provide a very important model system as they transport large quantities of water through gills that are highly vascular. Second, various species feed at differing trophic levels of the food chain, making a comparison among levels possible. Additionally, other species such as frogs, salamanders, turtles, and alligators or crocodiles could be used, depending on the ecological knowledge base for such species and the specific questions to be addressed. For example, many fish and amphibian species are short lived and are limited to specific water bodies whereas other fish, turtles, and alligators are long lived and may migrate between various wetland areas.

We would propose that a series of rivers, lakes, and marshlands be examined in Europe and North America. If possible, a few common species from similar genera should be examined to minimize interspecies variation. However, as the relative sensitivities of various species are unknown, this survey should examine species that occur in large numbers. Detailed sampling should be designed and similar protocols used. In addition to vitellogenin, plasma androgens and estrogens should be examined to ensure that natural estrogens are not elevated in those males that exhibit a positive response for plasma vitellogenin. These data would also provide supporting evidence of abnormal reproductive activity as previous studies have demonstrated abnormal steroidogenesis following exposure to various contaminants.

The limitation of this study, as presented, is the need for antibodies that recognize vitellogenin from the various species of interest. Vitellogenin is a large, globular protein, approximately 400 to 500 kD, with a complex, three-dimensional structure. Currently, a universal antibody that would cross-react with the vitellogenin of all oviparous species is not available but a number of laboratories are presently developing and screening possible candidates. Collaboration with these laboratories should be fostered. However, if a universal assay is not possible, other methods such as polyacrylamide gel electrophoresis (SDS-PAGE) can be used to detect plasma vitellogenin, although this technique would provide poor quantification of plasma concentrations as compared to a validated radioimmunoassay. An additional limitation of this survey involves the specificity of vitellogenin as an estrogen-induced product. A survey for vitellogenin will not detect possible antiandrogens or antiestrogens in the environment. Additional assays for other biomarkers may be required, although the plasma steroid analyses performed simultaneously with vitellogenin assays may provide clues to reproductive abnormalities due to other mechanisms of endocrine disruption.

Surveys need to be performed on a series of wetlands that represent a continuum in water quality. Sites with known contamination will serve as positive controls, whereas other localities with reduced input of manmade chemical contaminants can serve as negative controls. One point of caution should be made concerning the negative control localities—areas perceived as being pristine, such as arctic and antarctic regions, are known to have animal and human populations with elevated body burdens of various persistent environmental contaminants. Thus, it is important that representative species be examined from each test locality for persistent contaminants (e.g., DDE, total PCBs, dioxin). These measurements of contaminant load should not be used as a measure of possible estrogenic contamination, but rather as a general measure of possible environmental pollution. Initial surveys should examine as many male fish as possible (n=~100 males/wetland). Blood samples would be collected rapidly for analysis of vitellogenin, androgens, and estrogens. Specific body characteristics should be noted such as size, body weight, and other descriptive morphometric characteristics. For alligators or other wildlife having phallic structures, measurements of size and developmental abnormalities should be noted.

Experimental Studies —*in Vitro* and *in Vivo* Screening

The action of estrogenic chemicals, especially during fetal development in most species, is, in part, determined by their relative binding with intracellular targets and extracellular binding proteins. Estrogenic chemicals with relatively poor binding to extracellular proteins, such as SHBG and AFP, would be expected to have a disproportionate effect on developing estrogen target tissues. This is the case for DES. Experiments are proposed to evaluate the micropharmacokinetics of a selected group of priority environmental agents with estrogenic activity with regard to distribution within both the maternal and fetal compartments. Care must be given as to selection of the appropriate animal model with special regard to endogenous estrogen levels and binding components. As an example, an early step would be to study the relative binding *in vitro* of selected chemicals to SHBG, AFP, and the estrogen receptor.

To understand the etiology of estrogen-associated developmental abnormalities that may be related to later disease or dysfunction, it is crucially important to study the ontogeny of estrogen responsiveness in target tissues associated with reproduction in appropriate animal models. In addition to whole-animal studies, experiments should be attempted in organ and cell cultures of fetal genital and gonadal tissues; additionally, studies with transgenic mice would be illustrative. These studies should certainly include or emphasize the ontogeny and regulation of the estrogen receptor and related signaling molecules. This information would help to predict which chemicals would most likely alter sexual development and provide strategies for prevention.

Estrogenic chemicals can alter male reproduction through chronic or acute mechanisms. Long-term exposure to these chemicals during fetal/neonatal life can alter the expression of various other hormones, leading to impaired development of the testis such that the capacity for sperm production in adult life is irreversibly reduced. Direct effects on the adult testis that interfere with sperm production are also possible, although less likely. On the other hand, hormonally active xenobiotics can alter a process or expression of a gene during a short window of susceptibility that has long-lasting consequences. Characterization in detail of these later consequences is recommended. There are a few key genes involved in the development of the reproductive tract and gonads, and these provide important targets for study. For example, the effect of estrogen on the expression, regulation, and structure of *SRY*, Wilm's tumor gene (*WT-1*), *MIS*, *MIS* receptor, growth factor receptors and their ligands, estrogen receptor, genes for steroidogenic and steroid-metabolizing enzymes, and homeotic genes and related genes should be determined.

The class of chemicals called environmental estrogens comprises a diverse group of molecules. Studies to determine the metabolism, distribution, bioaccumulation, and excretion of representative members of this functional class in adult, pregnant, and fetal systems (both *in vivo* and *in vitro*) are proposed. This also includes identification of their active estrogenic forms. These data are important to make accurate exposure

estimates for susceptible target tissues in the human fetus.

The incidence of testicular cancer in humans is still increasing while its etiology is still unknown. A plausible hypothesis is that estrogenic exposure early in testicular development is associated with later germ cell neoplasia in humans. Studies to elucidate molecular and morphological markers for testicular development are needed. The cell type at risk for neoplastic transformation should also be studied and the process determined. Creative application of modern molecular biological and cell biological techniques should be considered to establish the malignant cell lines. Targeting germ cell-specific gene promoters for transgenic approaches to specific cell transformation and the development of new animal models to study should be encouraged. Studies on the physiology and pathophysiology of estrogens in developing and adult male germ cells are needed to evaluate the role of estrogens in testicular neoplasia. Attention should be given to new experimental models, such as the pig and nonhuman primates.

In order to improve the safety assessment of environmental chemicals, it should be assured that future toxicological testing used for the generation of safety data adequately takes into account the possible detrimental effects on male reproduction of chemicals possessing estrogenic and/or otherwise endocrine-disrupting activity. This probably necessitates the development of new test strategies. It is anticipated that the goal can be achieved by combining modifications of existing protocols for reproductive toxicity testing with the introduction of new *in vitro* and *in vivo* assays. The strategy should be based on insight into the mechanisms of action as currently known and emerging from future research activities. The development of improved methods should be an international collaborative effort to obtain worldwide acceptance of their possible use in regulatory safety assessments.

The exposure to possible xenoestrogens should be considered in far more detail than currently practiced in such studies. Methods should be elaborated that address the following questions:

- What chemicals are man (and wildlife) exposed to, by which routes, and to what degree?
- Do these chemicals actually get absorbed (they will not cause effects if they are not absorbed)?
- What are the concentrations of these chemicals in man (and wildlife)? Do any of them accumulate?
- How are these chemicals distributed in the body? Are they mobilized (redistributed) in certain states, such as pregnancy?
- How are they metabolized? Is it the metabolites, rather than the parent compounds, that are of concern? For example, alkylphenol polyethoxylates (a group of surfactants) are not estrogenic, but their degradation products are.
- If humans (and wildlife) are exposed simultaneously to a mixture of estrogenic, and/or antiestrogenic, antiandrogenic, or otherwise endocrine disrupting compounds, rather than to an individual chemical, would the overall effect be diminished or enhanced (additivity, synergism, antagonism)?

Presently we do not have adequate answers (or even preliminary answers in some cases) to any of these questions, mainly because we do not know what chemicals are of the greatest concern.

A major emphasis should be placed on how to identify priority compounds that justify examination (in addition to those already identified as estrogens). The aim would be to test for estrogenic activity of the chemicals to which man (and probably wildlife) is exposed the most.

The usefulness of various sources of information should be explored, such as existing product registers and OECD data on high-volume chemicals. In addition, advice and help may be obtained from industry, which may be able to assist in identifying the more important chemicals.

Collectively, these approaches should enable identification of the major man-made chemicals. These chemicals could then be screened to determine whether they possess any endocrine-disrupting activity. If no effect is observed, the significance of the compound (as far as this context is concerned) is likely to be trivial. If effects are observed, more detailed studies, including dose–response studies on animals, should be undertaken. If the results of these studies raise concern, further metabolic studies, such as accumulation in the body and access to the fetus, would provide further information and enable a better extrapolation to the human situation.

Conclusions

Male reproductive health has received remarkably little attention considering that subfertility affects 5% or more of men and that prostatic hypertrophy or cancer is a major problem for older men. It is now evident that several aspects of male reproductive health have changed dramatically for the worse over the past 30 to 50 years. The most fundamental change has been the striking decline in sperm counts in the ejaculate of normal men; recent evidence from Paris indicates that this decrease amounts to about 2% per year over the last two decades. The result is that many otherwise normal men now have sperm counts so low that their fertility is likely to be impaired. Over the last half-century, the incidence of testicular cancer has increased progressively in many countries to become now the most common cancer in young men. Other disorders of the male reproductive tract may also be increasing in incidence, with several European countries reporting a progressive rise in hypospadias (a malformation of the external genitalia) and an apparently emerging trend toward an increasing incidence of testicular maldescent.

These observations suggest that male reproductive health has declined progressively since the Second World War as a result of changes in environmental or lifestyle factors. While the etiologies underlying these apparent changes are currently unclear, both clinical and laboratory research suggests that all of the described changes in male reproductive health appear interrelated and may have a common origin in fetal life or childhood. This means that the increase in some of the disorders seen today originated 20 to 40 years ago, and the prevalence of such defects in male babies born today will not become manifest for another 20 to 40 years or more.

Trends in the reproductive health of species other than man also raise the possibility of environmental factors as partial etiologic contributions in a decline noted in male reproductive health of wildlife. For example, wild panthers in the United States have been reported to have an increase in undescended testes and a decrease in semen quality, whereas male alligators in some lakes in Florida have been shown to have abnormalities in their sex hormone levels (tending toward femaleness) and to have smaller than normal genitalia. Male fish in some parts of the United Kingdom have been shown to express a femalelike response when studied in a relatively natural setting. Earlier studies of fish-eating birds in the United States demonstrated nests containing male hatchlings that were apparently feminized. A recent report of lactating male fruit bats suggested that the males were, in some way, exposed to a female sex hormone. Recent laboratory

studies showed that when estrogenic forms of PCBs were painted on turtle eggs, the male hatchlings were sex-reversed to females. Taken together, this growing body of evidence suggests that environmental factors that resemble female sex hormones may be having an adverse effect on the reproductive capacity and well being of diverse species.

It has been well established that exposure of the male fetus to supranormal levels of estrogens can result in many, if not all, of the reproductive defects referred to earlier. Experiments in which potent synthetic estrogens, such as DES, were given to pregnant laboratory animals have demonstrated that prenatal exposure to synthetic estrogens results in a spectrum of adverse effects on the male offspring including undescended testes, testicular cancer, decreased semen quality, epididymal cysts, hypospadias, and poor fertility. Men similarly exposed *in utero* to DES have been reported to display related abnormalities such as cryptorchidism, lower sperm counts, cysts of the epididymis, and initially decreased fertility. The wealth of experimental results and associated clinical reports suggests strongly that prenatal exposure to exogenous estrogens may play an etiologic role in the trends observed in human male reproductive health.

The growing number of reports demonstrating that common environmental contaminants and natural factors possess estrogenic activity presents the working hypothesis that the adverse trends in human male reproductive health may be, at least in part, associated with exposure to estrogenic environmental chemicals during fetal and childhood development. The reproductive health trends in men are consistent with this hypothesis. While exposure levels to estrogenic chemicals are not at all well known for humans, the large number of chemicals in numerous environmental categories suggests adequate availability. For example, environmental chemicals reported to be estrogenic include, but are not limited to, some ubiquitous chlorinated hydrocarbons, such as PCBs and DDT; some products of detergent and surfactant manufacture, such as the alkylphenols; and some products released from plastics such as bisphenol-A and some phthalates. Many other compounds in our natural and synthetic environment demonstrate estrogenic activities and more are being discovered as the search continues. Although not the subject of this report, in considering and evaluating the possible role of estrogenic chemicals in male reproductive disorders, it should not be forgotten that many chemicals may have a detrimental effect on male reproductive health through mechanisms other than an estrogenic effect.

The reproductive health trends for men, the emerging data from wildlife, the well-controlled experimental data with developmental exposure to estrogens, and the less well-studied, but consistent, data on human cohorts, as well as the growing knowledge concerning hormonally active environmental chemicals, all point in the same direction. We conclude that these issues, taken *in toto*, indicate the need for a vigorous research effort to understand the extent of the problem, its underlying etiology, and the development of a strategy for prevention and intervention. Since the health research issues involved are complex and multifactorial and since these issues apparently involve research needs in many disciplines, countries and, indeed, species, this report outlines a multinational, interdisciplinary research effort involving academic, government, and industrial scientists and comprising field and clinical studies, epidemiological research, and basic and applied laboratory research with a comprehensive view to the relationships that will evolve. We consider that this approach will optimize the existing strengths of various institutions and investigators on a worldwide basis and will be the most cost-effective use of fiscal and intellectual resources.

There are many research needs in this area; a detailed description of these needs and strategies to fill them are considered in the full report. In addition to key basic, clinical, and epidemiological studies, the highest priorities go to investigations to fill crucial data gaps necessary to make informed decisions about the risk these chemicals may pose to human health. The most pressing areas of need are reliable estimates of exposure of humans to estrogenic chemicals at different ages including fetal life, and of how the exposures relate to adverse effects on the reproductive system; use of animal models to identify what levels of exposure to estrogenic chemicals do, and what levels do not, impair reproductive development; more penetrating studies of the molecular events that lead to impaired spermatogenesis and testicular cancer; improved studies on the structural and analytical chemistry of chemicals with special emphasis on the prediction of estrogenic activity from the molecular structure of a chemical; and the development of rapid *in vitro* and *in vivo* test systems for the detection of estrogenic potency of environmental chemicals and the harmonization of these screening tests with improved techniques for quick, reliable analysis of this chemical class. The proposed high-priority research will help provide the information necessary for appropriate risk assessments.

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Appendix A: Toxicological Evaluations of Pesticides and Environmental Chemicals

Regulatory Requirements for Safety Data on Pesticides and Other Chemicals

There is a principal difference between pesticides and other environmental chemicals. Pesticides are approved by the authorities for use in the production of food crops. This means that in a number of cases residues are accepted in food. However, the pesticide residues in foods are very closely regulated. This, of course, does not imply that pesticide residues are accepted in other media such as drinking water and air.

For the environmental pollutants, their presence in food is not intended but may be caused by many different factors, of which the contamination of food chains and migration/formation during storage and production are significant. It should be emphasized that although the uses of various highly persistent compounds such as PCBs and many chlorinated pesticides (e.g., DDT) have been severely restricted or banned for a number of years in many Western countries, they can still be found in foods, in particular in fat from fish and domestic animals. As a consequence of their persistence in the food chain, these compounds are also present in human tissues. Concentrations of organochlorine contaminants in human reproductive tissue, adipose tissue, and blood from the general population worldwide, as well as the concentrations in human breast milk fat, are presented in Tables A1 and A2, respectively [adapted from Thomas and Colborn (*1*)].

Approval by regulatory agencies of a pesticide demands that the health risk associated with exposure to the compound be evaluated by the regulating authorities. In Denmark, pesticides are approved for use by the Danish Environmental Protection Agency; and residues in foods are regulated through the establishment of maximal residue limits (MRL) for each single pesticide by the Danish National Food Agency. A substantial part of these evaluations takes place within the framework of the European Union. In brief, the MRLs are based on three parameters:

- A toxicological evaluation of the pesticide in question, which results in the establishment of an acceptable daily intake (ADI) for humans
- An evaluation of effects and residues of the pesticide in vegetable and animal food commodities after controlled application trials using the principles of Good Agricultural Practice (GAP)
- An evaluation of the possible intake of pesticide residues from treated food crops. In establishing the MRL for each food commodity, exceeding the ADI is not accepted.

Data from toxicological investigations are a substantial part of the assessment of a pesticide. The toxicological studies should identify possible adverse health effects of the compound and establish the dose at which such effects are likely to occur, and in particular identify a dose level where adverse effects are absent. The requirements for toxicological investigations have been developed since the early 1960s, especially during the international cooperation within the Codex Alimentarius of the FAO/WHO. ADIs and suggestions of MRLs are established at yearly meetings in a committee of independent experts, the so-called Joint FAO/WHO Meetings on Pesticide Residues (JMPR). A similar expert group, the Joint FAO/WHO Expert Committee on Food Additives establishes tolerable daily intakes of contaminants in foods. In the United States, U.S. EPA establishes reference doses (RfD) for pesticides and environmental chemicals using principles similar to those of JMPR.

The majority of the toxicological data used in the risk assessment are generated in studies performed according to internationally accepted standards (guidelines), which for each type of study state the minimum requirements for an acceptable performance. In addition, regulatory agencies also require that, to be used in decision making, the investigations are performed according to Good Laboratory Practice (GLP) involving quality control and quality assurance. The different types of toxicological investigations required for the evaluation of a pesticide involve studies on acute effects, including effects on skin and mucous membranes, and more important long-term studies on chronic effects, including carcinogenicity following repeated, daily exposure. Studies on effects on reproduction over a minimum of two generations are also required, as well as special studies on teratogenicity and embryo/fetotoxicity, and on effects on the genetic materials. In addition, studies on absorption, biotransformation, bioaccumulation, distribution, and excretion are also required. In these studies, effects on macromolecules, such as DNA, enzymes, and other biochemical parameters are often included.

The toxicological data that are used in the risk assessment are usually generated from animal experiments and *in vitro* investigations. When human data are available, for example from occupational or accidental exposure, these of course are considered highly valuable.

From the toxicological data a no observable effect level (NOEL) or a no observable adverse effect level (NOAEL) is identified as the highest daily dose level that does not produce observable effects or adverse effects in the most sensitive animal species. In establishing the ADI for humans the NOEL is reduced by a (un)safety factor, which takes into account the uncertainties of the results of the investigation, the extrapolation from animals to humans, and the variations in sensitivity and life-style within the human population. When the toxicological background material is considered sufficient, a safety factor of 100 is normally used (a factor of 10 for differences between species and 10 for differences within species). Additional safety factors are occasionally used, for example, when the biological effect is considered to be particularly serious or when uncertainty exists in the evaluation of the consequences of a finding. Safety factors of 1000 or even higher have occasionally been used.

When a clear NOAEL cannot be established on the basis of the available data, a lowest observed adverse effects level (LOAEL) is sometimes identified and used to establish an ADI. In this case, a safety factor larger than 100 is always used (the numerical value depends on the quality of the data and the severity of the effect).

The ADI covers the intake during the whole lifetime, including childhood, and is defined as the daily intake of the compound that—on the basis of all relevant information at that time—is considered not to result in adverse health effects. ADI is expressed in milligrams per kilogram of body weight (bw). The use of "considered not to" and "on the basis of all relevant information at that point in time" underlines that the toxicological investigations have their inherent limitations, that a zero-risk does not exist, and that the ADI may change in the light of new knowledge. ADI

Table A1. Concentration of organochlorine contaminants in human reproductive tissue, adipose tissue, and blood from the general population worldwide.[a]

Geographic location/year of sample/chemical	Number of samples (% positive)	Units/mean	SD	Range	Tissue	Case information[b]	Quantification analysis and detection limits
Norway, Oslo (1981–1982)						No history of exposure	
		ppb (μg/kg)			Maternal		
HCB	15 (100)	2	2		serum/wet	Caesarean	0.001 mg/kg (HCH)
	20 (90)	1	1		"	Normal delivery	
PCBs	15 (93.3)	10	4		"	Caesarean	0.002 mg/kg (PCBs)
	20 (100)	10	7		"	Normal delivery	
p,p'-DDE	15 (100)	19	21		"	Caesarean	0.002 mg/kg (DDE)
	20 (100)	10	8		"	Normal delivery	
β-HCH	15 (73.3)	<1			"	Caesarean	0.001 mg/kg (bHCH)
	20 (30)	<1			"	Normal delivery	
					Umbilical		
HCB	12 (83.3)	2	1		cord serum	Caesarean	
	20 (85)	1	2		"	Normal delivery	
PCBs	12 (75)	5	4		"	Caesarean	
	20 (65)	3	1		"	Normal delivery	
p,p'-DDE	12 (100)	10	9		"	Caesarean	
	20 (100)	3	2		"	Normal delivery	
β-HCH	12 (16.6)	<1			"	Caesarean	
	20 (10)	<1			"	Normal delivery	
Yugoslavia (1985–1986)		ppb (μg/l) Geometric			Maternal	No history of exposure	GLC-ECD
β-HCH	14 (100)	1.69		1.0–3.6	serum	Nonpregnant	
	14 (100)	1.45		1.1–2.8	"	At delivery	
γ-HCH	14 (100)	1.77		1.2–3.6	"	Nonpregnant	
	14 (100)	2.72		0.9–5.4	"	At delivery	
p,p'-DDE	14 (100)	9.31		6.1–14.4	"	Nonpregnant	
	14 (100)	7.57		2.8–11.7	"	At delivery	
p,p'-DDT	14 (100)	5.87		3.2–9.7	"	Nonpregnant	
	14 (100)	3.62		1.0–8.2	"	At delivery	
PCBs	14 (100)	2.86		1.8–3.9	"	Nonpregnant	
	14 (100)	2.01		1.1–4.65	"	At delivery	
Yugoslavia, North Adriatic area (1989)		ppb (μg/l) Median				No history of exposure	GC-ECD
HCB	10 (100)	2		1.0–4	Blood	Samples taken	
α-HCH	10 (80)	2		1.0–2	"	from lactating	
β-HCH	10 (80)	18		13.0–31	"	mothers	
p,p'-DDE	10 (100)	6		4.0–13	"		
PCB	10 (100)	7		6.0–?	"		
					"		
India, Bombay (1987)		ppm			" Amniotic fluid	No history of exposure	GC-ECD
PCBs	26 (100)	0.131	0.026	0.001–1.162	"		
					"		
					"	Male professionals	
(1986–1987)		ppm			"		
PCBs	60 (100)	0.837	0.01	0.005–3.33	Blood		
India, Lucknow (1979–1980)		ppb			Placental tissue	No history of exposure	GLC-ECD
HCH	27 (100)	39.9		10.7–97.9	"	Liveborn	
	9 (100)	35.7		17.2–62.4	"	Stillborn	
α-HCH (Lindane)	27 (100)	17.1		4.1–95.6	"	Liveborn	
	9 (100)	13.4		5.5–26.1	"	Stillborn	
Aldrin	27 (<100)	8		0.0–85.3	"	Liveborn	
	9 (<100)	31.7		0.0–83.3	"	Stillborn	
p,p'-DDE	27 (100)	18.3		2.8–93.0	"	Liveborn	
	9 (100)	12.4		4.7–22.3	"	Stillborn	
p,p'-DDT	27 (100)	13.8		2.0–46.3	"	Liveborn	
	9 (100)	38.5		5.4–80.0	"	Stillborn	
DDT total	27 (100)	39.8		7.6–162.2	"	Liveborn	
	9 (100)	60.8		21.9–93.2	"	Stillborn	

(continued)

Table A1. *(continued)*

Geographic location/year of sample/chemical	Number of samples (% positive)	Units/mean	SD	Range	Tissue	Case information[b]	Quantification analysis and detection limits
India, Delhi and Faridabad (1987)		ppb (ng/g)				No history of exposure	GLC-ECD
HCB	7 (42.9)	12.19	8	0–64	Adipose/wet		U.S. EPA Manual, 1980
HCB	4 (100)	280	280	59–830	Adipose/wet		
Poland, Pobnan (1981)		ppm (µg/g)				No history of exposure	GC-ECD
HCB	36 (86.1)	0.029	0.025	0.004–0.117	Testicular		
α-HCH	36 (100)	0.018	0.014	0.004–0.058	Testicular		
β-HCH	36 (72.2)	0.124	0.09	0.049–0.571	Testicular		
γ-HCH	36 (69.4)	0.022	0.02	0.004–0.083	Testicular		
Δ-HCH	36 (97.2)	0.055	0.053	0.011–0.254	Testicular		
ε-HCH	36 (38.8)	0.094	0.045	0.039–0.177	Testicular		
p,p'-DDE	36 (97.2)	0.072	0.045	0.015–0.216	Testicular		
Heptachlor epoxide	36 (8.3)	0.131	0.047	0.083–0.194	Testicular		
Massachusetts, New Bedford (1985–1986)		ppb				No history of exposure	
PCBs	391	5.9		0.0–60.9	Blood	Males	
	449	5.7		0.0–154.2	Blood	Females	
Michigan (1980–1981)		ppb (ng/ml)				Children, 4 year old—no history of exposure	GLC-ECD (Webb-McCall method)
PCBs	205 (52.2)	4.18	3.29	1.00–19.40	Blood	Fish exposure	3 ng/ml
	80 (48.8)	4.82	4.81	1.00–23.30	Blood	Farm exposure	3 ng/ml
PBBs	205 (12.7)	2.44	1.5	1.00–6.40	Blood	Fish exposure	1 ng/ml
	80 (21.3)	2.95	2.66	1.00–9.50	Blood	Farm exposure	1 ng/ml
DDT	202 (69.8)	4.36	3.87	1.00–21.40	Blood	Fish exposure	1 ng/ml
	79 (77.2)	4.24	4.47	1.00–143.2	Blood	Farm exposure	1 ng/ml
Missouri (1987)		ppt				No history of exposure	HRGC-HRMS
2,3,7,8-TCDD	50 (100)	54.4	125.8	2–745	Adipose/whole		
	50 (100)	0.519	1.314	0.013–8.290	Serum/whole		
Texas, El Paso (1984–1985)		ppb				No history of exposure	U.S. EPA , 1974 Manual of Analytical Methods
Aldrin	112 (34)	4.6	8.6	0.0–46.8	Blood		
BHC	112 (24)	2.5	2	0.0–16.5	Blood		
p,p'-DDE	112 (99)	7.1	4.3	0.0–34.6	Blood		
Heptachlor	112 (19)	3.1	3	0.0–9.9	Blood		
Texas, Gulf Coast (1979–1980)		ppm				No history of exposure	GLC-ECD
HCB	7 (85.7)	0.021		0.0–0.043	Adipose		0.005 ppm (86%)
p,p-DDE	7 (100)	3.849		0.843–6.527	Adipose		0.004 ppm (96%)
2,4,5,2',4',5'-HCBP	7 (100)	0.184		0.098–0.267	Adipose		0.005 ppm (92%)
2,3,4,2',4',5'-HCBP	7 (100)	0.645		0.211–1.625	Adipose		0.005 ppm (96%)
Germany, Munich		ppt				No history of exposure	HRGC/HRMS
T4CDD	28 (100)	8		2.6–18.0	Adipose		
P5CDD	28 (100)	16.4		7.7–40.4	Adipose		
H6CDD	28 (100)	94.7		35.7–178.2	Adipose		
H7CDD	28 (100)	106.7		35.1–246.0	"		
OCDD	28 (100)	373.2		116.5–789.1	"		
T4CDF	28 (100)	2.5		0.7–12.8	"		
P5CDF	28 (100)	35.2		7.6–93.3	"		

(continued)

Table A1. *(continued)*

Geographic location/year of sample/chemical	Number of samples (% positive)	Units/mean	SD	Range	Tissue	Case information[b]	Quantification analysis and detection limits
Germany, Munich *(continued)*							
H6CDF	28 (100)	41.5		15.8–146.0	"		
H7CDF	28 (100)	14.2		3.8–45.6	"		
OCDF	28 (100)	4		1.2–13.5	"		
T4CDD	28 (100)	16.4		1.0–88.9	Liver/fat		
P5CDD	28 (100)	20.2		7.3–58.7	"		
H6CDD	28 (100)	166.8		56.4–615.1	"		
H7CDD	28 (100)	1002.4		95.5–3463.1	"		
OCDD	28 (100)	4416.2		472.7–15259	"		
T4CDF	28 (100)	5 5		0 9–45 3	"		
P5CDF	28 (100)	173.7		36.7–643.0	"		
H6CDF	28 (100)	389.5		40.8–1800.7	"		
H7CDF	28 (100)	218.9		12.2–757.0	"		
OCDF	28 (100)	29.7		4.3–65.8	"		
Pakistan, Quetta						No history of exposure	GC/MS
(1987)	21 (66.6)	ppb (μ/l) 0.396		0–1.88	Blood		
α-HCH	21 (90)	1.99		0–7.16	"		
β-HCH	21 (100)	9.26		0.53–18.98	"		
4,4′-DDE	21 (76.2)	0.94		0–4.83	"		
4,4′-DDT							
Spain, agrarian area (1985–1987)		ppm (ml/kg)			Adipose/ext. lipid	No history of exposure	GLC-ECD
HCB	87 (100)	2.99	2.24		"		
p,p′-DDE	87 (100)	6.27	5.67		"		
Lindane	87 (100)	0.083	0.05		"		
β-HCH	87 (100)	3.06	5.18		"		
p,p′-DDD	87 (62)	0.079	0.079		"		
p,p′-DDT	87 (100)	1.5	0.89		"		
Dieldrin	87 (100)	0.072	0.068		"		
Australia, Sidney (1985–1986)		ppm (μg/g)				No history of exposure	GLC-ECD
	292 (99.3)	3.72		0.0–26.30	Adipose/wet		0.001 ppm (81%)
DDT	292 (99.3)	0.13		0.0–0.16	"		0.001 ppm (81–102%)
Dieldrin							
Canada, Ontario (1984)		ppb (ng/g)				No history of exposure	GC/MS
	141 (100)	84	56	18–373	Adipose		1.4 ng/g
HCB	141 (100)	84	82	14–530	"		3.0 ng/g
β-HCH	141 (100)	33	27	2–107	"		1.1 ng/g
Heptachlor	141 (100)	3237	2602	138–12167	"		1.2 ng/g
epoxide	141 (99.3)	47	41	0–235	"		0.9 ng/g
p,p′-DDE	141 (100)	84	80	7–369	"		1.7 ng/g
Dieldrin	141 (92.2)	11	13	0–98	"		1.8 ng/g
p,p′-DDT	141 (100)	2136	1473	197–11209	"		100 ng/g
Mirex							
PCB							
Israel, Jerusalem (1984–1985)		ppb (ng/g)				No history of exposure (males 5-year history of infertility= Study group)	GC-MS
	29	3.09	6.81	0.0–28.8	Blood	Study group	
p,p′-DDT	14	0.4	0.99	0.0–3.5	"	Control group	
	29 (100)	16.1	11.82	2.9–43.4	"	Study group	
p,p-DDE	14 (100)	10.55	8.17	2.1–32.1	"	Control group	
	29	7.111	6	0.0–21.6	"	Study group	
o,p′-DDT	14	5.83	8.6	0-0–32 2	"	Control group	
	29	2.61	3.51	0.0–15.4	"	Study group	

(continued)

Table A1. *(continued)*

Geographic location/year of sample/chemical	Number of samples (% positive)	Units/mean	SD	Range	Tissue	Case information[b]	Quantification analysis and detection limits
o,p-DDE	14	1.27	2.04	0.0–6.4	"	Control group	
	29	2.28	2.42	0.0–9.8	"	Study group	
Lindane	14	1.13	0.671	0.0–3.0	"	Control group	
	29	3.65	3.71	0.0–15.9	"	Study group	
Dieldrin	14	2.69	2.47	0.0–7.1	"	Control group	
	29 (100)	8.31	3.83	3.0–15.8	"	Study group	
Heptachlor	14 (100)	11.64	3.67	1 7.0–21.7	"	Control group	
epoxide	29	11.21	13.48	0.0–64.2	"	Study group	
	14	7.94	14.69	0.0–47.3	"	Control group	
Total PCBs							
Japan						No history of	GC-ECD
(1986–1988)		ppm				exposure	
β-HCH	23 (100)	0.84	0.44	0.37–2.02	Adipose/fat		
p,p'-DDE	23 (100)	2.4	2.5	11.04–0.52	"		
Heptachlor	23 (91.3)	0.07	0.06	0.00–0.25	"		
epoxide	23 (100)	0.67	0.48	0.13–2.16	"		
Chlordane	20 (85)	0.08	0.08	0.00–0.27	"		
Dieldrin							
Japan, Tokushima		ppb (ng/g)				No history of	GC-ECD
(1984–1985)		Geometric				exposure	
Chlordane	22 (100)	0.51	1.6	0.18–1.16	Blood	Male subjects	0.01 ppb
	21 (100)	0.46	1.7	0.12–1.12	"	Female subjects	
Japan						No history of	GC-MS
(1985)		ppt (pg/g)				exposure	
2,3,7,8-TCDD	13 (92.3)	9		6–18	Adipose/wet	(Cancer patients)	
1,2,3,7,8-PCDD	13 (100)	15		3–36	"		
1,2,3,4,7,8-HCDD	13 (69.2)	8		5–14	"		
1,2,3,6,7,8-HCDD	13 (92.3)	70		26–220	"		
1,2,3,7,8,9-HCDD	13 (76.9)	12		4–44	"		
1,2,3,4,6,7,9-HCDD	13 (69.2)	28		14–63	"		
1,2,3,4,6,7,8-HCDD	13 (92.3)	77		29–180	"		
08CDD	13 (92.3)	230		26–1100			
PCDDs total		410		160–1400	"		
2,3,7,8-T4CDF	13 (100)	9		3–12	"		
2,3,4,7,8-P5CDF	13 (100)	26		4–71	"		
1,2,3,4,7,8-/1,2,3,4,7,9-H6CDF	13 (84.65)	15		4–24	"		
1,2,3,4,7,9-H6CDF	13 (84.6)	14		3–28	"		
2,3,4,6,7,8-H6CDF	13 (23.1)	8		4–16	"		
PCDFs total		63		7–120	"		

Abbreviations: GC-ECD, gas chromatography–electron capture detector GLC-ECD, gas–liquid chromatography–electron capture detector GC-MS, gas chromatography–mass spectrometry HRGC-HRMS, high-resolution GC-MS. [a]Adapted from Thomas and Colborn (*1*). [b]General information regarding the subjects analyzed.

is not a danger-limit, but rather a safe value for an acceptable exposure to the pesticide in question. Exposures that lead to exceeding the ADI on a short-term basis are not expected to result in any health hazard.

GAP is defined as the nationally authorized safe method for application of a pesticide that under local conditions, such as climate, is found necessary for effective crop protection. The MRL is based on field spraying trials and subsequent determinations of residues. These investigations include different methods of application, including those using the highest dosages and used in such a way that the lowest possible amount of residue is produced. The MRL is never established at a level higher than needed even if the established ADI value would allow a higher residue content. In practice, this means that the intake of most pesticides by the general population is well below the ADI. In the evaluation of the health risk, the possible total intake of the pesticide is calculated as if the concentration in all the food in which it can be present is at the MRL for each single food item. This means that exceeding the MRL in one single sample does not automatically result in exceeding the ADI, as many samples normally are without detectable residues.

The toxicological evaluations of contaminants and industrial chemicals are in principle performed following the same principles that are used for the pesticides. However, as exposure to such compounds is not intended, but in many cases is unavoidable, tolerable daily intakes (TDIs) are established rather than ADIs.

For many environmental chemicals the toxicological database is less comprehensive than for most pesticides. This is true for many chemicals that were introduced

Table A2. Human breast-milk fat concentrations worldwide compared with the lowest observed endocrine or reproductive effect levels in animal studies. [a]

	Human data		Animal data	
Chemical and units	Average levels in human breast milk fat	High levels, location	Doses, exposure route	Impairment
Dieldrin	0.05 ppm	1.78 ppm Australia 1.00 ppm Iraq 1.00 ppm Uruguay	20 mg/kg/bw. Single injection to ten 3-week-old rats	Induced testoserone, 16α- and 16β-hydroxylases
Heptachlor and its epoxide	0.05 ppm	2.50 ppm Spain 0.48 ppm Italy	250 μmole/kg/bw. Single injection to 3-week-old rats	Induced testosterone, 16α- and 16β-hydroxylases
Chlordane (oxychlordane and *trans*-nonachlor)	0.08 ppm	>2.00 ppm Mexico and Iraq	0.16 mg/kg wt/day. Dietary exposure to pregnant mice throughout gestation	Elevation of plasma corticosterone concentrations, when measured on day 400 in both male and female offspring.
Total DDT	1.00 ppm	>100 ppm Guatemala		
o,p'-DDT	0.25 ppm		2 ppm. Dissolved in corn oil and injected into yolk of gull embryos	Caused feminization (a thickened ovarylike cortex in left testis).
HCB	0.10 ppm	7.00 ppm Greece	300 μmole/kg/bw. Single injection to 3-week-old rats.	Induced testosterone, 16α- and 16β-hydroxylases.
β-HCH	1.00 ppm	6.50 ppm Chile	50 mg/kg/bw (ppm). Dietary exposure to weaned rats for 13 weeks.	Caused reduction in testes weight.
γ-HCH	0.05 ppm	0.89 ppm Italy	8 mg/kg (ppm). ip daily in glycerine-suspension to adult male rats for 10 days.	Caused reduction in testes weight, degeneration of seminiferous tubules and affected both spermatocytes and spermatids
Total PCB	1.00 ppm	3.6 ppm Canada (Hudson Bay)	8 mg Aroclor 1254/kg (ppm). Dissolved in 0.2 ml peanut oil. Lactating dams were exposed via their diet on days 1,3,5,7, and 9 of lactation	Males exposed only during lactation exhibited reduced fertility as adults. Specifically, decreased number of implants and decreased number of embryos when mated normal females
2,3,7,8-TCDD	2.00 ppt	1.45 ppb Vietnam (1970s)	0.064 μg/kg (ppb)- Dietary exposure to pregnant rat on day 15 of gestation	Male offspring had decreased sperm counts when exposed perinatally *in utero* and via lactation

[a] Adapted from Thomas and Colborn) (*1*).

in the society before the stricter regulations of new chemicals were enforced in many countries during the 1970 to 1980s.

Toxicological Summaries on Pesticides and Other Chemicals Implicated as Environmental Hormones

The following summaries on the most important toxicological properties of various compounds that have been implicated as environmental hormones are mainly based on the monographs prepared by the expert groups of the JMPR and on the reviews performed by the U.S. EPA as published in the IRIS database (U.S. EPA's Integrated Risk Information System). In addition, valuable information can be found in the Environmental Health Criteria (EHC) documents published by the International Programme on Chemical Safety (IPCS). These monographs are published under the joint sponsorship of the United Nations Environment Programme, the International Labour Organisation, and the World Health Organization. Most attention has been paid to those compounds that have not already been discussed in "Environmental Chemicals with Known Estrogenic Effects" and "Exposure of Humans to Environmental Chemicals with Estrogenic Activity and Their Effects on Male Reproductive Health."

Preliminary estimates of dietary intakes are included (unpublished data). It is anticipated, but not necessarily true for all of the compounds, that diet is the major route of exposure for the general population. It is

known that the drinking water in Denmark meets the general limit standard of 0.1 fg/liter, and that for most of the compounds the levels of exposure are expected to be very low. However, for some of the herbicides, recent analysis of groundwater samples has in some cases revealed the presence of 2,4-dichlorophenoxyacetic acid (2,4-D) and atrazine near the limit value.

For pesticides and PCBs these preliminary estimates of dietary intakes are based mainly on the results and experiences of more than 30 years of pesticide control of Danish and imported foodstuffs. The results have been continuously published (in Danish) in reports from the Danish National Food Agency. The latest report was published in 1994, covering the results of the samples analyzed in 1993 (*2*). The latest sales statistics of the chemicals were published 1994 (*3*).

Herbicides

The exact exposure to the herbicides described later in this section is not known. To a limited extent, the herbicides have been included in the Danish pesticide control program since the early 1980s. When analyzed, these herbicides were found to be almost absent in food samples during the 1970s in spite of considerable use. Herbicides are used on plant food crops only very early in the growth season and are therefore less likely to be present in the final product. This view is supported by the observations of other countries. MRLs for the herbicides in fruits and vegetables have been set at the limit of detection, typically 0.05 mg/kg. The above-mentioned observations lead to the assumption that the average herbicide concentration in all fruits and vegetables, after rinsing, peeling, and preparating for consumption, is at least 1000 times lower than the MRL, e.g., less than 0.05 fg/kg. In Denmark the yearly intake of fruits and vegetables has been estimated at 60 kg per capita for each food category. This would correspond to an intake of less than 0.006 mg of each herbicide per person per year.

2,4-Dichlorophenoxyacetic Acid. 2,4-Dichlorophenoxyacetic acid and derivatives are currently being used as herbicides. The dietary intake estimate of less than 0.006 mg/person/year would correspond to a daily intake of less than 0.0000003 mg/kg bw/day for a 60-kg person. If it is assumed that drinking water contains 2,4-D at a level near the limit value of 0.1 fg/liter, and the daily consumption is set at 2 liters for a 60-kg person, a daily intake of 0.000003 mg/kg bw/day can be estimated. 2,4-D was most recently evaluated by JMPR in 1975 when an ADI of 0 to 0.3 mg/kg bw was established (*4*).

An RfD for noncarcinogenic effects of 2,4-D has been established by the U.S. EPA (*5*) at 0.01 mg/kg bw/day based on a no effect level of 1.0 mg/kg bw/day in rat subchronic bioassays. At 5.0 mg/kg bw/day, hematologic, hepatic and renal toxicities were observed. The safety factor was 100. Histopathological examinations correlated well with kidney organ weight changes showing cortical and subcortical pathology. Increases in ovarian weights and T4 levels were not considered to be treatment related. Increases were also noted in thyroid/pituitary weights at high dose levels; however, no histopathological changes were found (*5*). Chronic toxicity and reproduction studies of 2,4-D exposure indicated no adverse effects at dietary levels up to 500 ppm in dogs (approximately 14.5 mg/kg bw/day), up to 1250 ppm in rats (approximately 62.5 mg/kg bw/day) (*6*), or at levels of 1000 ppm in drinking water (50–100 mg/kg bw/day) in pregnant rats (exposed through gestation and for 10 months following parturition) or their offspring (exposed for up to 2 years after weaning) (*7*). 2,4-D does not accumulate in the mammalian organism. Further toxicological information on 2,4-D exposure can be found in EHC 29 (*8*) and EHC 84 (*9*).

2,4,5-Trichlorophenoxyacetic Acid. For 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), the dietary intake estimate of less than 0.006 mg/person/year would correspond to a daily intake of less than 0.0000003 mg/kg bw/day for a 60-kg person. The most recent evaluation of 2,4,5-T by JMPR is from 1981 when an ADI of 0 to 0.03 mg/kg bw was established (*10*).

An RfD for noncarcinogenic effects of 2,4,5-T was established by the U.S. EPA at 0.01 mg/kg bw/day and is based on a no effect level of 3 mg/kg bw/day in a 2-year feeding study in rats and a three-generation reproduction study in rats. At 10 mg/kg bw/day, increased urinary coproporphyrin was observed in the chronic study and a tendency toward reduced neonatal survival was seen in the three-generation reproduction study. The safety factor was 300 (*5*).

Other studies have also shown effects on reproduction in mice, rats, hamsters, and monkeys. A NOEL of 8 mg/kg bw/day for reduced fetal body weight was reported for NMRI mice (*11*). Fetal mortality was observed after administration of 2,4,5-T at 40 mg/kg bw/day (highest dose) to pregnant hamsters. Cleft palate was induced in A/JAX mice at 15 mg/kg bw/day; lower doses were not tested. Other strains of mice were less sensitive. Higher doses (approximately 200 mg/kg bw/day) induce frank teratogenic effects in rats. A qualitative association between 2,4,5-T exposure and human birth defects has been suggested. Terata and fetotoxic effects have not been observed in monkeys up to a dose of 40 mg/kg bw/day (*5*). 2,4,5-T does not accumulate in the mammalian organism.

Alachlor. For alachlor, the dietary intake estimate of less than 0.006 mg/person/year would correspond to a daily intake of less than 0.0000003 mg/kg bw/day for a 60-kg person. Alachlor has not been evaluated by JMPR.

An RfD for noncarcinogenic effects of alachlor was established by the U.S. EPA at 0.01 mg/kg bw/day based on a no effect level of 1 mg/kg bw/day in a 1-year dog-feeding study. At 3 mg/kg bw/day, hemolytic anemia was observed; and in an earlier study using higher dose levels, changes indicative of liver toxicity were recorded. The safety factor was 100. No signs of testicular toxicity were observed in any of these studies (*5*).

In a 2-year chronic toxicity/carcinogenicity study of alachlor in rats, a NOEL for systemic toxicity was found at 2.5 mg/kg bw/day. At 15 mg/kg bw/day, molting of retinal pigmentation, increased mortality rate in females, and abnormal disseminated foci in the liver of males were seen. In an earlier 2-year feeding study in rats, 14 mg/kg bw/day and higher dose levels resulted in degenerative ocular and hepatic changes as well as other pathological gross and microscopic findings in the thyroid, kidneys, brain, spleen, heart, prostate, and ovaries (*5*).

In a three-generation reproduction study of alachlor in rats using dietary levels of 0, 3, 10, and 30 mg/kg bw/day, no significant adverse effects on the reproductive performance of adult rats were observed at any dose tested. Compound-related effects were seen on kidneys (increased weights and pathological changes in parents and progeny) in the high-dose males and females. Lower ovary weights were noted in the high-dose females of each parental generation and in pups from the third generation. The NOEL for systemic toxicity was 10 mg/kg bw/day (*5*).

In a developmental toxicity study in rats, soft stools, hair loss, anogenital staining, and maternal death were observed after 400 mg/kg bw/day, whereas the

NOEL for maternal toxicity was 150 mg/kg bw/day. Based on a slight decrease in mean fetal body weight and a slight increase in mean postimplantation loss, the NOEL and LOAEL for developmental toxicity were 150 and 400 mg/kg/day, respectively. In the rabbit, the NOEL for developmental toxicity was found to be equal to or greater than 150 mg/kg bw/day (*5*).

Amitrole. No residues of amitrole have been detected in food after recommended use (*12*). The previously mentioned preliminary dietary intake estimate of less than 0.006 mg/person/year would correspond to a daily intake of less than 0.0000003 mg/kg bw/day for a 60-kg person.

Amitrole was evaluated for acceptable daily intake by JMPR in 1993, when a temporary ADI of 0 to 0.0005 mg/kg bw was established based on a no effect level of 0.5 mg/kg bw/day in a long-term toxicity/carcinogenicity study in rats. At a dose level of 5 mg/kg bw/day, an increase in the incidence of thyroid tumors and an increase in (mainly benign) pituitary tumors were observed. Due to the noncompleteness of the available reproduction study, a safety factor of 1000 was used (*13*).

In mice, rats, and sheep, but not in Syrian hamsters, dogs, or cattle, amitrole affects the thyroid. In the rat, a no effect level was established at 2 ppm in the diet for 6 to 13 weeks (equivalent to 0.1 mg/kg bw/day) based on increased iodine uptake (shortly after injection), increased thyroid weight, and histopathological changes of the thyroid (goiter and clearly activated thyroids). The mechanism involves inhibition of thyroid peroxidase, resulting in decreases in circulating levels of T_4 and T_3, which stimulate the pituitary to increase secretion of thyroid-stimulating hormone (TSH), which in turn may cause thyroid hypertrophy, hyperplasia, and neoplasia.

Long-term toxicity/carcinogenicity studies have been performed in mice, rats, and golden hamsters. In the mouse, liver and thyroid tumors were observed after administration of 1000 mg/kg bw/day by gavage. In a special carcinogenicity study, in which pups were treated for a period of 90 weeks at a level of 500 ppm in the diet, a slight increase in liver tumors was observed. In another 18-month carcinogenicity study in mice, levels of 0, 1, 10, or 100 ppm in the diet did not increase the incidence of tumors. At 100 ppm an increase in thyroid weight was observed.

In the rat, 100 ppm in the diet for 24 months produced an increase in the incidence of thyroid tumors and an increase in (mainly benign) pituitary tumors. At this dose level the thyroid weight was increased throughout the study. There was no effect with 10 ppm in the diet. Amitrole was considered not to be teratogenic in the rat at dose levels up to 1000 mg/kg bw/day. Slight maternal toxicity and fetal and embryotoxicity (reduced fetal body weight/litter, reduced skeletal ossification and increased incidence of enlarged and/or dark fetal thyroids) were seen after dose levels of 500 and 1000 mg/kg bw/day.

In the hamster, up to 100 ppm of amitrole in the diet produced no evidence of carcinogenicity, and no effect was observed on the thyroid.

In the rabbit, embryo and fetotoxicity were observed after a dose level of 40 mg/kg bw/day. The no effect level was considered to be 4 mg/kg bw/day.

No adequate reproduction study is available. From a limited study in rats at dietary concentrations ranging from 25 to 1000 ppm, effects on reproductive capability were observed at a dose level of 500 ppm and above. Reduction of liver weight and thyroid hyperplasia was observed at the lowest level studied, 25 ppm (equivalent to 1.3 mg/kg bw/day).

Amitrole does not accumulate in the mammalian organism. Additional toxicological information can be found in EHC 158 (*12*).

Atrazine. For atrazine, the preliminary dietary intake estimate of less than 0.006 mg/person/year would correspond to a daily dietary intake of less than 0.0000003 mg/kg bw/day for a 60-kg person. If it is assumed that the drinking water contains atrazine at a level near the limit value of 0.1 fg/liter, and the daily consumption is set at 2 liters for a 60-kg person, a daily intake of 0.000003 mg/kg bw/day can be estimated. Atrazine has not been evaluated by JMPR.

An RfD for noncarcinogenic effects of atrazine was established by the U.S. EPA at 0.035 mg/kg bw/day based on a no effect level of 3.5 mg/kg bw/day in a 2-year rat feeding study. Mean body weights were significantly depressed in males and females receiving 25 and 50 mg/kg bw/day. The absolute weight of liver and kidney in males receiving 50 mg/kg bw/day was significantly lower than that in controls. The safety factor was 100 (*5*). Acinar hyperplasia of the mammary gland and epithelial hyperplasia of the prostate were increased in males receiving 50 mg/kg bw/day when compared to controls. In females receiving 25 or 50 mg/kg bw/day, there were increases in the incidences of mammary fibroadenomas and adenocarcinomas.

In beagle dogs administered atrazine for 1 year in the diet, a dose level of 4.97 mg/kg bw/day was without toxicity. At a level of 33.65 mg/kg bw/day, clinical signs and pathological changes related to cardiac toxicity were found (*5*).

Atrazine is able to induce hormonal imbalances in rodents. Most work has been directed toward the effects of atrazine on the pituitary–gonadal axis. Steroid hormone metabolism was found to be impaired by atrazine. At 120 mg/kg bw/day for 7 days, atrazine increased the wet weight of the anterior pituitary (hyperemia and hypertrophy) and reduced the activities of several steroid-metabolizing enzymes in the rat. The deethylated metabolite of atrazine was equally potent. Prenatal treatment with atrazine or deethylatrazine (16 mg/kg bw, sc) did not alter pituitary metabolism in male pups, but atrazine increased 5-α-steroid reductase activity in the female pups. Treatment pre- and postnatally with atrazine and its metabolite decreased 3-α-hydroxysteroid dehydrogenase activity, and atrazine decreased 5-α-steroid reductase activity in the male pups. Both compounds decreased the number of androgen-specific binding sites in the prostate. Neither compound had any effect on female pituitary androgen metabolism. *In vitro* studies have demonstrated inhibition of rat pituitary androgen metabolism by atrazine (*14*).

In a three-generation reproduction study with atrazine in rats, body weights were significantly lower for high-dose animals (34.97 mg/kg bw/day) throughout the study. Body weight gains were also significantly depressed at the high dose. The NOEL for parental toxicity was 3.5 mg/kg bw/day. At 34.97 mg/kg bw/day an equivocal decrease in body weights of the second generation male pups was observed. Therefore, the NOEL for reproductive toxicity was also 3.5 mg/kg bw/day (*5*).

Several developmental toxicity tests have been performed in rats and rabbits. In these studies, embryotoxicity and fetotoxicity have been observed only at dose levels where maternal toxicity was also evident. In addition, developmental toxicity studies in rats of two metabolites of atrazine, hydroxyatrazine and diaminochlortriazine, did not reveal a specific potential concerning embryo- or fetotoxicity (*5*).

Atrazine does not accumulate in the mammalian organism.

Metribuzin. For metribuzin the dietary intake estimate of less than 0.006

mg/person/year would correspond to a daily intake of less than 0.0000003 mg/kg bw/day for a 60-kg person. Metribuzin has not been evaluated by JMPR.

An RfD for noncarcinogenic effects was established by the U.S. EPA at 0.025 mg/kg bw/day based on a no effect level of 2.5 mg/kg bw/day in a 2-year dog-feeding study. At 37.5 mg/kg bw/day mortality, decreased body weights and liver and kidney effects were observed. The safety factor was 100 (*5*).

In a 2-year chronic toxicity/carcinogenicity study in rats, the NOEL was 5.0 mg/kg bw/day. Following a dose level of 15.0 mg/kg bw/day, decreased body weight gain and pathologic changes in the liver, kidneys, uterus, and mammary glands were observed.

Metribuzin was not carcinogenic to rats. Enlarged thyroids were reported in male rats administered 25 and 75 mg/kg bw/day for 3 months (*5*). Metribuzin was not carcinogenic in a 2-year mouse study. At 480 mg/kg bw/day, changes were observed in hematological parameters and liver weight. The NOEL was 120 mg/kg bw/day.

Studies on developmental toxicity in rats and rabbits have not revealed teratogenicity at doses up to 100 or 15 mg/kg bw/day, respectively. In a three-generation reproduction study in rats, a NOEL of 15 mg/kg bw/day for reproductive effects and maternal toxicity was established (*5*).

Metribuzin does not accumulate in the mammalian organism.

Trifluralin. For trifluralin, the dietary intake estimate of less than 0.006 mg/person/year would correspond to a daily intake of less than 0.0000003 mg/kg bw/day for a 60-kg person. Trifluralin has not been evaluated by JMPR.

An RfD for noncarcinogenic effects of trifluralin was established by the U.S. EPA at 0.0075 mg/kg bw/day based on a no effect level of 0.75 mg/kg bw/day in a 1-year dog-feeding study. At 3.75 mg/kg bw/day, increased liver weights and an increase in methemoglobin were observed. The safety factor was 100 (*5*).

At higher dose levels administered to the dog for 6 months (10 mg/kg/day), enlarged livers, discolored kidneys, corneal vascularization, hemolytic anemia, and increased alkaline phosphatase concentrations were seen (*5*).

Several subchronic and chronic bioassays in the mouse and rat have produced NOELs that are higher than the one observed in the dog (*5*). In a 3-month feeding study in rats, the pituitary weight relative to the body weight was decreased after 40 mg/kg bw/day.

In two two-generation reproduction studies in the rat, the lowest NOEL for reproductive effects was identified at 32.5 mg/kg bw/day. At 100 mg/kg bw/day reduced litter size was seen. In the parents, increased kidney weights and renal lesions of the proximal tubules were observed after doses of 32.5 mg/kg bw/day. Teratology studies in rats showed no malformations, whereas fetotoxicity (decreased mean fetal body weight) was observed after doses of 1000 mg/kg bw/day, a dose level that also produced maternal toxicity. However, in another teratology study in rats, reduced skeletal maturity and increased vascular fragility were reported after doses of 20 mg/kg bw/day. In the rabbit, no terata were observed, whereas fetotoxicity (decreased fetal weight and increased number of fetal runts) was seen at a maternally toxic dose of 500 mg/kg bw/day (*5*).

In one 2-year chronic bioassay of trifluralin using F344 rats, significant increases were found in the incidences of bladder papillomas and renal pelvis carcinomas at the highest dose level tested in female and male rats. In addition, a significant increase in the incidence of follicular cell tumors of the thyroid gland (adenomas and carcinomas combined) occurred at the highest dose tested in male rats. Four other rodent chronic bioassays of trifluralin in the diet have been performed. These included a 2-year study in Sprague-Dawley rats, a 78-week study in Osborne-Mendel rats, a 78-week study in B6C3F1 mice, and a 2-year study in B6C3F1 mice. Trifluralin did not produce significant increases in the incidence of tumors in any of these studies (*5*).

Fungicides

Benomyl (and Its Principal Metabolite Carbendazim). Benomyl is widely used as a fungicide together with carbendazim. In both instances the active principle is methyl-2-benzimidazolecarbamate and MRLs are expressed as carbendazim.

The main source of exposure for the general population is the residue of benomyl and carbendazim in food crops. Dietary exposure analysis in the United States and the Netherlands yielded an expected mean daily intake of about one-tenth the ADI for benomyl and carbendazim (0.001–0.002 mg/kg bw/day) (*15*). In 1993, residues were found in 1 of 72 samples of apples and 1 of 30 samples of bananas in Denmark. The detection limit is 0.1 mg/kg, which is also the MRL in most crops. However, in some fruits the MRL is 2 mg/kg. Considering that residues were found in only a few samples and that intake of residues primarily will originate from fruits, it is assumed that the average concentration in all fruits, after rinsing, peeling, and preparing for consumption, is at least 1000 times lower than the MRL, e.g., less than 0.002 mg/kg. In Denmark the yearly intake of fruits has been estimated at 60 kg per capita. This implies an intake of less than 0.120 mg of carbendazim per person per year corresponding to a daily intake of less than 0.000006 mg/kg bw/day for a 60-kg person.

In 1983, JMPR established an ADI of 0 to 0.02 mg/kg bw for benomyl. In 1985, an ADI was established of 0 to 0.01 mg/kg bw for carbendazim. These ADI values were based on no effect levels for benomyl of 30 mg/kg bw/day for teratology studies in the rat and 125 mg/kg bw/day for chronic studies in the rat. The no effect level for carbendazim was 2.5 mg/kg bw/day in subchronic dog studies (*16–18*).

An RfD for noncarcinogenic effects of benomyl was established by the U.S. EPA at 0.05 mg/kg bw/day based on a no effect level of 5 mg/kg bw/day in a three-generation reproduction study in rats. At 25 mg/kg bw/day, decreased pup weaning weights were observed. The safety factor was 100 (*5*).

Toxicological monographs on benomyl and carbendazim have also been published by IPCS (*15,19*).

In the reproductive system, benomyl and its metabolite carbendazim cause, after an initial testicular swelling, a decrease in testis and epididymis weight in adult male rats, a reduction in caudal sperm reserves, a decrease in sperm production, and a decrease of male fertility. At higher doses, there is generalized disruption of all stages of spermatogenesis. Benomyl does not affect copulatory behavior, seminal vesicles, sperm mobility, or related reproductive hormones. The lowest benomyl concentration shown to induce statistically significant spermatogenic effect in male rats was 45 mg/kg bw/day. The NOEL for these effects was 15 mg/kg bw/day (*15,19*). The testicular effects of benomyl treatment in the adult rat were shown to be reversible after a recovery period of 70 days.

In one study in which prepubertal male rats (33 days of age) were administered 200 mg benomyl/kg bw/day for 10 days, no effects on sperm parameters and testicular histopathology were induced (*15*).

Several studies in rats and mice have demonstrated teratogenicity of benomyl and carbendazim. A NOEL of 30 mg/kg bw/day for benomyl has been established in the rat (*5*).

Benomyl and carbendazim caused liver tumors in two strains of mice that have a high spontaneous rate of liver tumors, whereas carbendazim did not produce tumors in a mouse strain with a low spontaneous incidence of tumors. Carcinogenicity studies with both compounds in the rat were negative (*10,20*).

Benomyl and carbendazim do not accumulate in the mammalian organism.

Ethylenebisdithiocarbamates: Mancozeb, Maneb, Metiram, and Zineb. Ethylenebisdithiocarbamates (EBDCs) are fungicides primarily used in the production of fruits and vegetables. Residues are frequently found at levels below the MRLs, which typically are between 0.5 mg (the detection limit) and 2 mg/kg. If a concentration of 1 mg/kg is used as an average MRL for fruits and vegetables, and it is assumed that the average concentration in all fruits and vegetables, after rinsing, peeling and preparing for consumption, is at least 100 times lower than the MRL, an intake of less than 1.2 mg per person per year can be obtained from 60 kg of fruits and 60 kg of vegetables. This would correspond to a daily intake of less than 0.00006 mg/kg bw/day for a 60-kg person.

These compounds were evaluated for acceptable daily intake by JMPR in 1993. A group ADI of 0 to 0.03 mg/kg bw was established for each compound alone or in combination because of *a*) the similarity of chemical structure of the EBDCs; *b*) the comparable toxicological profiles of the EBDCs based on the toxic effects of ethylenethiourea (ETU), which forms part of the terminal residue to which consumers of products treated with the EBDCs are exposed; and *c*) the fact that parent EBDC residues cannot be differentiated using presently available analytical procedures (*13*).

These compounds are rapidly absorbed, extensively metabolized, and rapidly excreted (90%) within 24 hr in rodents. ETU is the major metabolite.

The data on mancozeb would support an ADI of 0 to 0.05 mg/kg bw, based on a no effect level of 4.8 mg/kg bw/day in a rat long-term toxicity/carcinogenicity study using a safety factor of 100.

In the rat, mancozeb affects the thyroid. In a 13-week study in rats, the no effect level of mancozeb was 125 ppm in the diet, equal to 7.0 mg/kg bw/day, based on increased serum TSH and decreased T_4 values at 250 ppm in the diet.

Mancozeb was not carcinogenic in the mouse in two long-term toxicity/carcinogenicity studies. At the highest dose used, 1000 ppm in the diet (equivalent to 130 mg/kg bw/day) decreased body weight and decreased T_3 and T_4 values were recorded. In a two-year long-term toxicity/carcinogenicity study in the rat, a no effect level of 125 ppm in the diet, equivalent to 4.8 mg/kg bw/day, was observed. At 750 ppm in the diet, decreased body weight gains, decreased T_3 and T_4 values, increased thyroid weights, thyroid follicular cell hypertrophy, hyperplasia, and nodular hyperplasia were seen. Increased incidences of thyroid follicular cell adenomas and/or carcinomas were noted at this dose level.

In two 2-generation reproduction studies, the overall no effect level was observed at 120 ppm in the diet, equivalent to 7.0 mg/kg bw/day. At 150 ppm decreased body weights were seen and at 1200 ppm microscopic changes were observed in the thyroid, kidney, and pituitary gland. Other changes found were increased relative weights of liver, kidney and thyroid; decreased gestation and lactation, body weight, and feed consumption; decreased premating body weight and feed consumption.

Several teratogenicity studies in rats and rabbits have produced embryo and fetotoxicity only at dose levels where maternal toxicity was clearly evident. At a high dose level of 512 mg/kg bw/day, teratogenic effects were seen (*13*).

The data on maneb would support an ADI of 0 to 0.05 mg/kg bw, based on the no effect level of 5.0 mg/kg bw/day for thyroid effects in rat studies using a safety factor of 100.

In the rat, dog, and monkey, maneb affects the thyroid. In a 13-week study in rats, the no effect level of maneb was 80 ppm in the diet equal to 5.0 mg/kg bw/day, based on an increase in absolute thyroid weight and thyroid follicular cell hyperplasia at 400 ppm. In two studies in dogs, follicular cell hyperplasia in the thyroid was observed after 400 ppm in the diet for 13 weeks and 1000 ppm in the diet for 52 weeks, respectively. The overall no effect level was 200 ppm, which is equivalent to 6.4 mg/kg bw/day. In the monkey an increased thyroid weight was observed after 300 ppm in the diet for 6 months, while 100 ppm in the diet, equivalent to 7.3 mg/kg bw/day, was without effect.

Maneb produced hepatocellular adenomas in the mouse in a 79-week long-term toxicity/carcinogenicity study at a dose level of 2400 ppm in the diet. A no effect level was established at 60 ppm in the diet, equivalent to 11 mg/kg bw/day, whereas decreased body weight and decreased thyroxin levels were observed at 240 ppm.

In a 31-month long-term toxicity/carcinogenicity study in the rat, a no effect level of 300 ppm in the diet, equivalent to 20 mg/kg bw/day, was observed. At 1000 ppm in the diet decreased body weight, decreased T_4 values, and increased thyroid weights were seen. There was no evidence of carcinogenicity.

In two two-generation reproduction studies, the no effect level was observed at 75 ppm in the diet, equivalent to 5.6 mg/kg bw/day. At 300 ppm, increased organ to body weight ratios for liver and kidney, and follicular cell hyperplasia of the thyroid were seen.

Two teratogenicity studies in rats produced embryo fetotoxicity (increased early resorptions, increased postimplantation losses and a decrease in viable fetuses) only at dose levels at which maternal toxicity was clearly evident (*13*).

The data on metiram would support an ADI of 0 to 0.03 mg/kg bw based on a no effect level of 2.5 mg/kg bw/day for thyroid effects in dogs and the no effect level of 3.1 mg/kg bw/day in the long-term rat study, using a safety factor of 100.

In the mouse, rat, dog, and monkey, metiram affects the thyroid. In two 13-week studies in rats, the no effect levels of metiram were 80 and 100 ppm in the diet, equivalent to 5.8 and 6.0 mg/kg bw/day, respectively, based on decreased serum T_4 and an increase in absolute thyroid weight at 300 and 960 ppm, respectively. In dogs, follicular cell hyperplasia of the thyroid was observed after 1000 ppm in the diet for 52 weeks. The no effect level was 80 ppm, equivalent to 2.5 mg/kg bw/day. In the monkey, decreased serum T_3 and T_4 levels, increased thyroid weights, and minimal follicular cell hyperplasia of the thyroid were observed after 15 and 75 mg/kg bw/day administered by gavage for 26 weeks, whereas 5 mg/kg bw/day was without effect.

Metiram was not carcinogenic to the mouse in an 88-week long-term toxicity/carcinogenicity study at dose levels up to 1000 ppm in the diet. A no effect level was established at 300 ppm in the diet, equal to 24 mg/kg bw/day, whereas decreased body weight was observed at 1000 ppm.

In a 111-week long-term toxicity/carcinogenicity study in the rat, a no effect level of 80 ppm in the diet, equivalent to 3.1 mg/kg bw/day, was observed. At 320 ppm in the diet, muscular atrophy was observed. There was no evidence of carcinogenicity.

In a three-generation, two litter per generation reproduction study in rats, no adverse effects were revealed on reproductive parameters. The no effect level was observed at 40 ppm in the diet, which was equivalent to 1.8 mg/kg bw/day. At 320 ppm decreased parental body weight and food consumption were recorded in the F_0 and F_1 generations.

Metiram was not teratogenic in rats and rabbits at any dose tested during critical periods of organogenesis. The no effect levels for embryo/fetotoxicity were 80 mg/kg bw/day in the rat based on slight decreases in litter size and weight after dose levels of 160 mg/kg bw/day, and 40 mg/kg bw/day in the rabbit, based on decreases in mean fetal weights at 120 mg/kg bw/day. At both these dose levels maternal toxicity was evident (*13*).

In the rat and dog, zineb affects the thyroid. Dietary administration of zineb to rats for 6 weeks indicated a no effect level of 500 ppm, equivalent to 25 mg/kg bw/day, based on morphological changes of the thyroid gland and reduced iodine uptake at 5000 ppm. Rats treated by gavage for 4 weeks exhibited slight hyperplasia of the thyroid at a dose level of 1000 mg/kg bw/day, resulting in a no effect level of 250 mg/kg bw/day. In a limited study in dogs, follicular cell hyperplasia of the thyroid was observed after 10,000 ppm in the diet for 52 weeks, whereas 2000 ppm had no effect.

Zineb was not carcinogenic when given to mice at a dose of 460 mg/kg bw/day from postnatal day 7 until weaning, followed thereafter with dietary administration of 1300 ppm until 18 months of age. In a two-year study in rats, dietary levels of 500 ppm and higher revealed goitrogenic effects. At or above 1000 ppm renal congestion, nephritis, and nephrosis increased mortality and diminished growth rate. There was no evidence of carcinogenic potential. Neither of these two studies is considered adequate for the study of long-term toxicity/carcinogenicity according to the modern standard.

Treatment of rats with zineb at doses of 50 to 960 mg/kg bw/day suggested adverse effects on the reproduction. Sterility, decreased fertility, and resorption of fetuses were observed. From the limited data available, a dose level of 50 mg/kg bw/day appeared to be without significant adverse reproductive effects.

Zineb was not teratogenic in mice at dose levels up to 2000 mg/kg bw/day during critical periods of organogenesis, nor were maternal or embryo/fetotoxicity observed. The no effect level for teratogenicity and embryo/fetotoxicity was 630 mg/kg bw/day in the rat. At the maternally toxic dose level of 2000 mg/kg bw/day, zineb was teratogenic, resulting in a significant increase in hydrocephalus and skeletal anomalies (*13*).

Hexachlorobenzene. The use of hexachlorobenzene (HCB) as a seed dressing to prevent fungal disease on grains was discontinued in most countries in the 1970s. HCB continues to be released into the environment as a byproduct and contaminant in many other chlorinated chemicals including chlorinated solvents. HCB is highly stable in the environment and accumulates in the food chains. Humans are almost exclusively exposed through the diet, especially meat, fish, and poultry; and HCB accumulates in human adipose tissue. In Denmark 0.1 ppm of HCB was found in the fat from mothers' breast milk in 1986 (*21*). Typical concentrations found in various foodstuffs of animal origin are around 0.01 mg/kg fat. Similar values are found for fish. From the continuous surveillance of HCB in food, a maximal intake of less 0.73 mg per person per year has been estimated. This corresponds to an intake of less than 0.000033 mg/kg bw/day.

In 1978, JMPR withdrew the previous ADI of 0 to 0.0006 mg/kg bw/day (*22*).

An RfD for noncarcinogenic effects was established by the U.S. EPA at 0.0008 mg/kg bw/day based on a no-effect level of 0.08 mg/kg bw/day in a 130-week chronic feeding study in rats. The study involved feeding male and female rats diets containing HCB for 90 days prior to mating and until 21 days after parturition (at weaning), whereafter the offspring that had been exposed to HCB and metabolites *in utero* and from maternal nursing received the compound in their diets for the remainder of their lifetime (130 weeks). At a dose level of 0.3 mg/kg bw/day, liver changes were observed. At higher doses increases in pup mortality, hepatic toxicity including increased liver porphyrin levels, and severe chronic nephrosis (males only) were observed. The safety factor was 100 (*5*). HCB-induced porphyria has been reported for all species examined, except the dog.

Long-term dietary exposure of humans to HCB caused an epidemic of porphyria cutanea tarda (PCT) in Turkish citizens who accidentally consumed bread made from grain treated with HCB. In children less than 1 year of age, pink sore disease was observed along with 95% mortality. In addition to the PCT-associated symptoms of skin lesions, hypertrichosis, and hyperpigmentation, the exposure caused neurotoxicity and liver damage. Follow-up studies reported PCT symptoms, reduced growth and arthritic changes in the appendages of children who were directly or indirectly (i.e., through breast milk) exposed. Accurate exposure data (dose and duration) are lacking (*5*). HCB is a potent inducer of hepatic microsomal enzymes. Several studies have indicated that HCB affects the immune system.

A number of investigations have indicated that repeated exposure to HCB can affect male reproduction in various species, but only at relatively high doses. Thus, 30 mg/kg bw/day for 21 days reduced serum testosterone levels in male mice, probably due to induction of hepatic microsomal enzymes. Decreased fertility was observed in male rats after 250 mg/kg bw/day for 5 days. Histologic changes in the testes, leading to retarded maturation has been noted in the pig after 50 mg/kg bw/day for 90 days. In female monkeys HCB caused degenerative changes in the reproductive tissues. In female rats HCB produced elevated serum progesterone levels and no estrogenic effects were observed.

In a four-generation rat reproduction study, reduced litter sizes, increased number of stillbirths, and reduced pup survival were observed after a dose of 4 mg/kg bw/day. A NOAEL was established at 1 mg/kg bw/day.

Oral administration of HCB has induced tumors in the liver, thyroid, parathyroid, and kidney in three rodent species.

HCB was negative in most *in vitro* and *in vivo* mutagenicity assays (*5*).

Tributyltin Compounds. Tributyltin compounds were previously used in large quantities in marine antifouling paints on ships, boats, and mariculture pen nets. However, their use is now restricted in most countries.

Exposure to tributyltin compounds is not well documented; however, the dietary exposure is expected to be indeed very low.

In 1991, JMPR established an ADI of 0 to 0.0005 mg/kg bw for tributyltin compounds (fentin compounds) based on a no-effect level of 0.5 mg/kg bw/day in

short-term rat studies with respect to general, as well as specific, effects on the immune system (*23,24*).

An RfD for noncarcinogenic effects of tributyltin compounds was established by the U.S. EPA at 0.00003 mg/kg bw/day based on a no effect level of 0.025 mg/kg bw/day in a chronic feeding study in rats. At 0.25 mg/kg bw/day, an effect on host resistance to *T. spiralis* was reported. The safety factor was 1000 (*5*).

In a parallel lifetime carcinogenicity/chronic toxicity study in rats, the only observed immunological alterations were an increase of IgM and a decrease of IgG titers in the females at a dose of 2.5 mg/kg bw/day after 12 and 24 months of treatment. No effect was observed after a dose of 0.25 mg/kg bw/day (*5,24*). Similar immunological effects have been observed in short-term rat studies. In a 4-week feeding study, thymic atrophy was reported at dietary concentrations as low as 1 mg/kg bw/day. In a companion study, rats fed 1 or 4 mg/kg bw/day in the diet for at least 6 weeks displayed impaired cell-mediated immunity (*5*).

Short-term studies suggest that tributyltin decreases the activity of the pituitary–thyroid axis. Structural effects were observed in the pituitary and thyroid glands, and changes in circulating hormones were observed. However, in long-term studies most of these effects seemed to be absent. The mechanism of action is not known (*5,24*).

Tributyltin was found to be fetotoxic and teratogenic in mice and rats at a level at which overt maternal toxicity was also observed (18 mg/kg/day), with no effects observed at 9 mg/kg/day (*5*).

One carcinogenicity study in rats reported increased incidences of benign tumors of the pituitary gland (not dose related) and adrenal medullary tumors (pheochromocytomas) at a high dose. These tumor types appear usually at high and variable background incidences, and the significance of the finding is questionable (*24*).

Dicarboximides (Vinclozolin). Vinclozolin (3-(3,5-dichlorophenyl)-5-ethenyl-5-methyl-2,4-oxazolidinedione) is used as a fungicide on fruits, vegetables, vines, and ornamental plants and grasses. Residues slightly above the Danish MRLs of 1 to 2 mg/kg are occasionally found in some fruits and vegetables. If a concentration of 2 mg/kg is used as an average MRL for fruits and vegetables and it is assumed that the average concentration in all fruits and vegetables, after rinsing, peeling, and preparing for consumption is at least 100 times lower than the MRL, an intake of less than 2.4 mg per person per year can be obtained from 60 kg of fruits and 60 kg of vegetables. This would correspond to a daily intake of less than 0.00012 mg/kg bw/day for a 60-kg person.

Vinclozolin was evaluated for acceptable daily intake by JMPR in 1986 (*25*), where a temporary ADI of 0 to 0.04 mg/kg bw was estimated based on a no effect level of 7 mg/kg bw/day in a 6-month dog feeding study. A safety factor of 200 was used. The ADI was made temporary as 3,5-dichlorophenylcarbamoyl-2-propionic acid was identified as a major plant metabolite that had not been fully evaluated with respect to its toxicity, and additional studies were requested. In 1988 (*26*) JMPR established an ADI of 0 to 0.07 mg/kg bw for vinclozolin and all metabolites containing the 3,5-dichloroaniline moity, as additional data ensured the low acute toxicity and absence of mutagenicity of this plant metabolite. The safety factor was 100.

An RfD for noncarcinogenic effects of vinclozolin was established by the U.S. EPA at 0.025 mg/kg bw/day based on an estimated no effect level of 2.5 mg/kg bw/day from the same dog study used by JMPR. The safety factor was 100 (*5*).

After absorption vinclozolin is rapidly and extensively biotransformed and excreted as conjugated metabolites. The major metabolite found in rat urine was *N*-(3,5-dichlorophenyl)-2-methyl-2,3,4-trihydroxybutanoic acid amide (*25*).

Vinclozolin has a low order of acute toxicity and was negative in a number of *in vitro* and *in vivo* mutagenicity assays.

In a 6-month feeding study, groups of dogs were given daily dosages of vinclozolin equal to 0, 7.0, 20, 41, and 135 mg/kg bw/day (males) or 0, 7.4, 21, 41, and 141 mg/kg bw/day (females). Hemolytic anemia was observed in high-dose males and females, while other clinical chemistry parameters were not affected. Dose-related increases in absolute and relative weights of adrenals were noted in animal feed with dose levels of 20 mg/kg bw/day or higher. Other organ weight changes were noted, including decreased relative pituitary weights in females at dose levels of 21 mg/kg bw/day or higher. Upon microscopic examination, changes related to the anemia observed at the highest dose were recorded and, in addition, atrophy/stromal proliferation of the prostate (males) and vacuolization of the zona fasciculata of the adrenals (males and females) were seen. The no effect level was 7.0 mg/kg bw/day.

Vinclozolin did not show carcinogenicity in long-term studies in rats and mice after doses as high as 278 mg/kg bw/day (rats) and 912 mg/kg bw/day (mice). In the long-term mouse study, increases in absolute and relative weights of the liver were noted in females at the two highest dose levels, 287 and 912 mg/kg bw/day. Increased liver and testes weights were seen in male mice at the highest dose level of 818 mg/kg bw/day. After a complete microscopic examination no toxicologically significant differences were observed between treated and control animals. The no effect level was 87 mg/kg bw/day. In the long-term rat study, absolute weights of several organs were decreased at the two highest dose levels. The incidence of macroscopic and microscopic findings were randomly distributed among all test groups and did not appear to be related to treatment with vinclozolin. The no effect level was 27 mg/kg bw/day.

Special studies in mice and rabbits did not reveal any teratogenic potential of vinclozolin. In the mouse study it was found that mice receiving 6000 ppm or higher in their diets did not have any implantations. The no effect level was 110 mg/kg bw/day.

In a three-generation rat reproduction study, no effects were observed on alteration in fertility, gestation length, litter size, sex ratio of pups, fetal birth weight, weight gain of pups, or survival of pups. No treatment-related malformations or developmental defects were noted, and no treatment-related pathological findings were seen in either parent rats or pups at necropsy. The NOAEL was estimated to be higher than the highest dose tested, 152 mg/kg bw/day (*25*).

In contrast, recent reproduction/toxicity studies conducted in rats have shown Leydig cell tumors and atrophic ventral prostate and seminal vesicles after chronic exposure of adult male rats to vinclozolin. In addition, anogenital distance in male offspring was reduced to a femalelike size (*27*). Subsequently, another toxicological study (*28*) showed strong demasculinizing effect of vinclozolin in the male offspring of mice treated with 100 or 200 mg/kg bw/day from gestational day 14 to postnatal day 3; in addition to the femalelike anogenital distance many other abnormalities were observed, such as retention of nipples, hypospadias, epididymal granulomas, maldescent of the testis, and frequent

presence of vaginal pouch. These findings suggested that vinclozolin possesses antiandrogenic activity. Recent biochemical studies have demonstrated that the demasculinizing effects of vinclozolin may be attributed to two intermediary metabolites, 2-(3,5-dichlorophenyl)-carbamoyl)oxy-2-methyl-3-butenoic acid (M1) and 3′,5′-dichlo-2-hydroxy-2-methylbut-3-enanilide (M2), which inhibit the androgen receptor function, although in certain conditions these metabolites may act as agonists (*29,30*).

Insecticides

A number of the insecticides mentioned below are no longer approved for use. However, several of the compounds are very persistent and can be found in the environment as general background pollution. Therefore, residues may still be found in foods. In addition, residues may be contained in foods imported from countries where some of the compounds are still in use. In particular, the organochlorine pesticides are bioaccumulating in the food chains and are found widely distributed in fat-containing foods of mammalian or marine origin. In contrast to this environmentally based contamination of foods, it is characteristic that the development of several different types of new insecticides during the last few years has resulted in a shift to more specific and limited uses of the single approved insecticide. This means that residues of each single insecticide are not widespread but rather found in a limited number of crops.

β-HCH, dieldrin, heptachlor, heptachlor epoxide, and lindane (γ-HCH) are included in the Danish pesticide control program. With a few exceptions they are never found in fruits and vegetables, including cereals. Typical concentrations in fat of animal and marine species is about 0.01 mg/kg of fat. The intake from heavy consumption of animal fat and fish is estimated at less than 0.7 mg per person per year. This would correspond to a daily intake of less than 0.00003 mg/kg bw/day for a 60-kg person.

Chlordane and oxychlordane, methoxychlor, mirex, and toxaphene are not included in the Danish pesticide control program. These compounds are not used in Denmark. Therefore, the knowledge on exposure is rather limited. From concentrations reported in human mothers' milk (*21*), it seems reasonable to assume, that the exposures to these compounds are not higher than to the previously mentioned insecticides. Thus, a daily intake of less than 0.00003 mg/kg bw/day for a 60 kg person is assumed.

Carbaryl. Carbaryl is approved as an insecticide for use on plants and fruit trees. Food is considered the major exposure route for carbaryl. Residue findings ranging from trace to 0.05 mg/kg food have been reported. In United States the intake in 1969 was estimated at 0.003 mg/person/day (*31*). The MRL for cereals is 0.5 mg/kg. Assuming a concentration of 0.5 mg/kg as an average for fruits and vegetables, and assuming that the average concentration in fruits and vegetables after rinsing, peeling, and preparing for consumption is at least 100 times lower than the MRL, an intake of less than 0.6 mg per person per year can be obtained from 60 kg of fruits and 60 kg of vegetables. This would correspond to a daily intake of less than 0.00003 mg/kg bw/day for a 60-kg person.

Carbaryl was last evaluated by JMPR for acceptable daily intake in 1973. An ADI of 0 to 0.01 mg/kg bw has been established based on the following no effect levels: 10 mg/kg bw/day in the rat; 1.8 mg/kg bw/day in the dog; and 0.06 mg/kg bw/day for cholinesterase (ChE) inhibition in humans (*31,32*).

An RfD for noncarcinogenic effects was established by the U.S. EPA at 0.1 mg/kg bw/day based on a no effect level of 9.6 mg/kg bw/day in a chronic feeding study in rats. At a dose of 15.6 mg/kg bw/day, kidney and liver toxicity was seen. The safety factor was 100 (*5*).

Carbaryl has been shown to affect mammalian reproduction and perinatal development adversely in a number of species. Effects include impairment of fertility, decreased litter size, and reduced postnatal viability. Malformations have also been observed. Adverse reproductive and developmental effects were noted only at doses that caused maternal toxicity. Data indicate that the reproductive and developmental processes of mammals are not especially sensitive to carbaryl compared with the susceptibility of the adult organism (*31*).

In the mouse, doses of carbaryl up to 35 mg/kg bw/day for 5 days did not affect weights of testes and accessory sex glands or uptake of progesterone by the prostate gland. However, at 68 mg/kg bw/day, increased hepatic androgen hydroxylase activity was indicated (*31*).

There are conflicting data about effects of carbaryl on sperm counts and changes in sperm morphology in plant workers. No adverse effect on reproduction have been reported (*31*).

The results of numerous carcinogenicity studies in mice and rats have mainly been negative. However, these studies are old and do not meet modern standards. In the majority of genotoxicity assays, carbaryl did not have any DNA-damaging properties (*31*).

Malathion. The use of malathion is rather limited in food crops. No residues were reported in 1993 when a limit of 1 mg/kg food was used. If it is assumed that residues are present in 1/12 of fruits and vegetables and assuming that the average concentration in the food items after rinsing, peeling and preparing for consumption is at least 1000 times lower than 1 mg/kg, an intake of less than 0.01 mg per person per year can be obtained from 10 kg of fruits and vegetables. This would correspond to a daily intake of less than 0.0000005 mg/kg bw/day for a 60-kg person.

Malathion was last evaluated by JMPR for acceptable daily intake in 1966. An ADI of 0 to 0.02 mg/kg bw was established (*33*).

An RfD for noncarcinogenic effects was established by the U.S. EPA at 0.02 mg/kg bw/day based on a no effect level of 0.16 mg/kg bw/day in a subchronic human feeding study. At a dose of 0.34 mg/kg bw/day, depression of red blood cell cholinesterase activity was seen. The safety factor was 10 (*5*).

In a 2-year chronic toxicity/carcinogenicity study in rats, a NOEL of 5 mg/kg bw/day was observed. At a dose of 50 mg/kg bw/day, decreased brain cholinesterase and body weight were reported.

In a reproduction study in rats, reduced number of live pups and reduced pup body weight were seen at a dose of 240 mg/kg bw/day, the only dose tested. No teratogenic effect was seen in the rat after ip injection of 900 mg/kg bw/day (*5*).

Methomyl. Methomyl is approved for use on apples, pears, and some berries. Residues found in GAP trials are from 0.5 to 1.0 mg/kg raw food. In considering the reduction after rinsing, peeling, and preparing for consumption, it is assumed that the average concentration in fruits is at least 1000 times lower than 1 mg/kg, and that only 1/6 of fruits are contaminated. This would correspond to an intake of less than 0.01 mg of methomyl per person per year. The daily intake would then be 0.0000005 mg/kg bw/day for a 60-kg person.

Methomyl was evaluated by JMPR for acceptable daily intake in 1989. An ADI of 0 to 0.03 mg/kg bw was established (*34*).

An RfD for noncarcinogenic effects was established by the U.S. EPA at 0.025 mg/kg bw/day based on a no effect level of 2.5 mg/kg bw/day in a 2-year feeding study in dogs. At 10 mg/kg bw/day, kidney and spleen toxicity was seen. Histopathologic changes were observed in the liver and bone marrow at 50 mg/kg bw/day. The safety factor was 100 (*5*).

The NOEL observed in the dog study is further supported by lifetime studies in rats and mice, and a three-generation reproduction study in rats where higher NOELs were seen (*5*).

Dicofol. In 1993 Dicofol residues were found in a limited number of apples, citrus fruits, and berries at levels below the reporting limit of 1 mg/kg. If it is assumed that residues are present in 50% of fruits and assuming that the average concentration in fruits after rinsing, peeling, and preparing for consumption is at least 100 times lower than 1 mg/kg, an intake of less than 0.3 mg per person per year can be obtained from 60 kg of fruits. This would correspond to a daily intake of less than 0.000015 mg/kg bw/day for a 60-kg person.

Dicofol was evaluated by JMPR for acceptable daily intake in 1992. An ADI of 0 to 0.002 mg/kg bw was established based on the no effect level of 0.22 mg/kg bw/day in a rat long-term study. At 2.2 mg/kg bw/day, histopathological changes in the liver and vacuolation of adrenal cortical cells were observed (*35*).

Dicofol is structurally related to DDT, and therefore tends to accumulate in the mammalian organism, however at a rate much less than that of DDT and its metabolites.

Dicofol primarily affects the liver in experimental animals. It is an inducer of the hepatic mixed-function oxidase (MFO) activity in rats and mice. In the rat, an increase in the incidence of thyroid follicular cell hypertrophy in one study could not be reproduced in other studies. In the dog, a NOEL was found to be 0.82 mg/kg bw/day (1-year study) based on liver changes and reduced cortisol response to ACTH at 5.7 mg/kg bw/day (*35*).

Dicofol increased the incidence of liver adenomas in male mice at high doses, but not in female mice. Dicofol was not carcinogenic in the rat.

In a two-generation reproduction study in rats, the NOEL was 0.5 mg/kg bw/day based on increased incidences of ovarian stromal cell hyperplasia and hepatocellular changes at 2.5 mg/kg bw/day. Viability of offspring was reduced at higher doses. The NOEL for reproductive parameters was 2.1 mg/kg bw/day.

Dicofol was not teratogenic in mice, rats or rabbits. Embryotoxicity and fetotoxicity were observed at maternally toxic dose levels (*35*).

Methoxychlor. The dietary intake of methoxychlor has been estimated at less than 0.00003 mg/kg bw/day for a 60-kg person.

Methoxychlor was last evaluated by JMPR for acceptable daily intake in 1977. An ADI of 0 to 0.1 mg/kg bw was established (*20*).

An RfD for noncarcinogenic effects was established by the U.S. EPA at 0.005 mg/kg bw/day based on a no effect level of 5.01 mg/kg bw/day in a rabbit teratology study. At 35.5 mg/kg bw/day, excessive loss of litters due to maternal toxicity was seen. The safety factor was 1000 (*5*).

Teratology studies in rats as well as a three-generation reproduction study in rats and a 2-year chronic rat study all showed no effect levels higher than that observed for the rabbit.

Methoxychlor is considered to have estrogenic activity. Kupfer and Bulger (*36*) found that both methoxychlor and metabolites have estrogenlike activity with several metabolites having proestrogen activity.

Gray et al. (*37*) investigated the effects of methoxychlor on the pubertal development and reproductive function in the male and female rat by dosing rats from gestation, weaning, lactation, through puberty with either 25, 50, 100, or 200 mg/kg bw/day of methoxychlor. In females, an acceleration of vaginal opening, abnormal estrous cycle, inhibition of luteal function, and a blockage of implantation was observed. In males, an inhibition of somatic growth and accessory sex gland weight, elevated pituitary and serum prolactin levels, and a suppression of testicular Leydig cell function were found. Some of these effects occurred at levels as low as 25 mg/kg bw/day. These observations are consistent with the earlier reports that methoxychlor mimics estrogen both *in vivo* and *in vitro.*

Goldman et al. (*38*) investigated the subchronic effects of methoxychlor on the rat (Long-Evans hooded) reproductive system by dosing for 8 weeks with 25 or 50 mg/kg of methoxychlor by oral gavage. No effect was observed on the pituitary weight, serum LH, FSH, or prolactin levels and the pituitary LH or FSH concentrations. Pituitary prolactin levels were increased at both dose levels. There was an increase in GnRH levels in the mediobasal hypothalamus at the high-dose level. The authors determined that the reproductive effects of methoxychlor are mediated in part by an increase in prolactin release, which in turn influences the hypothalamic levels of GnRH. This may be considered an early effect of methoxychlor on the rat reproductive system.

Cummings and Gray (*39*) found that methoxychlor affects the decidual cell response of the rat uterus, suggesting a direct effect of the compound on the uterus with no effects on uterine weight, serum progesterone levels, or corpora lutea maintenance. Long-term exposure to methoxychlor reduced fertility and induced fetotoxicity. The effects of reduced fertility and fetotoxicity were noted in a three-generation reproduction study. Although the available data from these three studies are limited, it is apparent that methoxychlor at 1000 ppm produced reproductive effects in the form of reduced fertility index, reduced litter size, and reduced viability index.

Bal (*40*) reported inhibition of spermatogenesis, degeneration of spermatogonia and spermatocytes, and cytoplasmic vacuolation in the epithelium of the epididymis in male rats after the administration of 100 to 200 mg/kg bw/day methoxychlor. A decrease in seminal vesicle and caudal epididymal weight and caudal sperm count as well as delayed puberty were observed in neonatal rats administered 25 to 200 mg/kg bw/day methoxychlor for one generation, indicating that the endocrine function of the testes and pituitary gland were affected (*37*). Cooke and Eroschenko (*41*) also noted that the development of the neonatal male rat reproductive tract was inhibited by methoxychlor administration, as shown by a decrease in serum testosterone levels and decreased DNA content of the seminal vesicles, bulbourethral glands, and the ventral prostate. Rats fed 2000 ppm methoxychlor for 90 days exhibited decreased prostate size and cell content (*42*). Goldman et al. (*38*) hypothesized that part of methoxychlor's effects on male reproductive function may be mediated by a prolactinemic effect, since administration of 25 or 50 mg/kg/day methoxychlor to 21-day-old male rats caused an increase in serum prolactin levels and an increase in hypothalamic gonadotropin-releasing hormone levels.

Several chronic oral carcinogenicity bioassays have been conducted with methoxychlor, the results of which have been equivocal.

A number of studies show that methoxychlor does not accumulate in the body

to any appreciable degree, but accumulation of methoxychlor in fat has been observed following administration of very high dietary levels. Methoxychlor is metabolized in the liver to readily excretable polar compounds. The results of several studies indicate that methoxychlor does not induce hepatic microsomal enzymes (*5*).

Lindane (γ-Hexachlorocyclohexane or γ-HCH). The intake of lindane has been estimated at less than 0.00003 mg/kg bw/day for a 60-kg person.

Lindane tends to accumulate in body fat of mammalian species. More than 90% of the intake of lindane is estimated to originate from foods. Studies of the total diet in the United States estimated the intake up to 0.00005 mg/kg bw/day in 1970 and up to 0.000003 mg/kg bw/day in 1980 (*43*).

Lindane was last evaluated by JMPR for acceptable daily intake in 1989. An ADI of 0 to 0.008 mg/kg bw was established based on the following no-effect levels: 0.75 mg/kg bw/day in the rat; 1.6 mg/kg bw/day in the dog (*34,43*).

An RfD for noncarcinogenic effects was established by the U.S. EPA at 0.0003 mg/kg bw/day based on a no effect level of 0.33 mg/kg bw/day in a chronic feeding study in rats. At 1.55 mg/kg bw/day, kidney and liver toxicity was seen. The safety factor was 1000 (*5*).

In a 2-year bioassay in dogs, a NOEL for liver effects was determined to be 1.6 mg/kg bw/day. Lindane is a well-known inducer of the liver MFO system. Lindane induced hyperplastic nodules and/or hepatocellular adenomas in mice, especially in males given high doses in long-term studies. Lindane did not increase the incidence of liver tumors in rats (*43*).

Lindane had no teratogenic effect in mice, rats, dogs, and pigs. Fetotoxic and/or maternal toxic effects were observed with doses of 10 mg/kg bw/day and above. A dose of 5 mg/kg bw/day was considered a no effect level. In a three-generation reproduction study in rats, lindane had no effect on reproduction or maturation at doses up to 5 mg/kg bw/day. At 2.5 mg/kg bw/day, liver changes indicative of MFO enzyme induction occurred in the offspring of the third generation. The no effect level in this study was 1.25 mg/kg bw/day (*43*).

When given to pregnant mice at various stages of pregnancy, lindane (3.6–10.4 mg/kg bw/day) caused reproductive failure and fetotoxicity. The effects were partly corrected by co-administration of estrogen, while progesterone had no effect. When estrogen and progesterone were simultaneously given to pregnant mice that were fed lindane during early pregnancy, the fetal development became normal. It thus appeared that lindane caused a hormone deficiency, resulting in reproductive and developmental failure (*44*).

β-Hexachlorocyclohexane. β-Hexachlorocyclohexane (β-HCH) is a byproduct in the manufacture of lindane. β-HCH has been found in the air over oceans at 0.004 to 0.13 ng/m^3. β-HCH is the most persistent HCH isomer; bioconcentration takes place in invertebrates, fish, birds, and man. In mammals the compound is stored in adipose fat (*45*).

Food is the main exposure route for the general population. Total dietary studies from United Kingdom in 1966 to 1967 found 0.003 mg/kg, in 1975 to 1977 0.0005 mg/kg, and in 1981 <0.0005 mg/kg of total diet. The average daily intake in the United States in 1982 to 1984 ranged from less than 0.0000001 to 0.0000004 mg/kg bw/day for various age groups (*45*).

β-HCH is included in the Danish pesticide control program. As mentioned above, the intake has been estimated at less than 0.00003 mg/kg bw/day for a 60-kg person.

No ADI or RfD has been established for β-HCH. The most sensitive organ to the toxicity of β-HCH is the liver. In the rat, hypertrophy and proliferation of smooth endoplasmic reticulum and increased activity of microsomal enzymes were seen after a dose 2.5 mg/kg bw/day for 90 days. After 12.5 mg/kg bw/day, changes in the gonads (testicular atrophy) were reported, but associated hormonal changes showed no consistent endocrine effect. There were no adverse effects reported after 0.1 mg/kg bw/day. In a long-term study, liver enlargement and histological changes were seen at 0.5 mg/kg bw/day (*45*). In a two-generation reproduction study in rats, parental liver effects were also reported. No effects on reproduction were seen after 0.1 mg/kg bw/day, whereas 0.5 mg/kg bw/day resulted in increased mortality and infertility. The parental animals at 2.5 mg/kg bw/day showed decreased weight of ovaries and increased weight of adrenal glands and uterus in females. In males, the testes showed a reduced number of Leydig cells. A weak estrogenlike effect after a dose of 25 mg/kg bw/day for 5 days has been described in female mice and rats. The uterus was the target organ, and there were no clear effects on endocrine control systems. β-HCH did not displace 17β-estradiol from its receptors. The mechanism of the effect is unclear.

Positive or marginally positive tumorigenic responses, characterized as benign hepatomas or hepatocellular carcinomas, have been observed in two strains of mice (*45*).

DDT and Its Metabolites, DDE and DDD. The use of DDT in the Western world is now severely restricted. However, it is still extensively used in some developing countries against malaria. Technical DDT contains mainly the *p,p'*-isomer (75–80%); *o,p'*-DDT constitutes 10 to 25% of the technical product. The DDT metabolite DDE is more persistent than the parent compound, and is therefore the form of DDT normally found in foods and human tissues. Following the severe restrictions in the use of DDT in the late 1960s, a dramatic decline has occurred in the contamination of foods and humans. For example, levels in herring have declined from 5 to 7 mg/kg fat in 1973 to 0.5 to 1 mg/kg fat in 1993 (*2*). In 1986, DDE levels in human milk in Denmark ranged from 0.29 to 1.21 mg/kg fat (*21*).

DDT and its metabolites are included in the Danish pesticide control program. In 1993, the typical levels found in animal and fish lipids were about 0.02 mg/kg. It has been estimated that an individual who consumes a large amount of fish is exposed to less than 2.5 mg of DDT and its metabolites per year. This corresponds to less than 0.0001 mg/kg bw/day.

JMPR in 1984 established an ADI at 0 to 0.02 mg/kg bw/day for any combination of DDT, DDD, and DDE (*17*).

An RfD for noncarcinogenic effects was established by the U.S. EPA at 0.0005 mg/kg bw/day based on a no effect level of 0.05 mg/kg bw/day in a 27-week rat feeding study. At 0.25 mg/kg bw/day, liver lesions (hepatocellular hypertrophy) were observed. The safety factor was 100 (*5*).

In a three-generation rat reproduction study, offspring mortality increased at all dose levels, the lowest of which corresponds to about 0.2 mg/kg bw/day. Three other reproduction studies (rat and mouse) showed no reproductive effects at much higher dose levels (*5*).

Nine feeding studies, including two multigenerational studies, have been conducted in mice. Only one of these studies, conducted for 78 weeks, showed no indication of DDT tumorigenicity. However, both hepatocellular adenomas

and carcinomas were observed in six mouse studies. Benign and malignant lung tumors were observed in two studies in which mice were exposed both *in utero* and throughout their lifetime. Doses leading to the increased tumor incidence ranged from 0.15 to 37.5 mg/kg bw/day. Three studies using rats and doses from 25 to 40 mg/kg bw/day produced an increased incidence of benign liver tumors. Another study was negative, as were three additional assays in which lower doses were given (*5*).

Studies of DDT exposure in hamsters have not shown an increased tumor incidence. Unlike mice and humans, hamsters accumulate DDT in tissue but do not metabolize it to DDD or DDE. Studies of DDT in dogs and monkeys have not shown a carcinogenic effect. However, the length of these studies was insufficient to assess the carcinogenicity of DDT (*5*).

p,p'-DDE exposure increased the incidence of liver tumors including carcinomas in males and females of two strains of mice and in hamsters. In the rat, a dose-dependent, but not statistically significant, increase in thyroid tumors in females was not considered convincing evidence of carcinogenicity (*5*). DDE was mutagenic in mouse lymphoma cells and Chinese hamster (V79) cells, but not in *Salmonella.*

p,p'-DDD induced an increased incidence of lung tumors in male and female mice, liver tumors in male mice, and thyroid tumors in male rats (*5*).

Chlordane, Heptachlor, and Heptachlor Epoxide. At one time, these insecticides were widely used in the United State, but now the use of chlordane is suspended, and that of heptachlor restricted. Technical chlordane contains a mixture of various chlordane isomers and heptachlor (chlorinated cyclodienes). Oxychlordane and heptachlor epoxide are persistent metabolites of chlordane and heptachlor, respectively.

Levels of heptachlor/heptachlor epoxide in human milk fat in the 1970s ranged from 0.10 to 0.35 ppm in Europe and America, whereas chlordane and oxychlordane levels are low and mostly below the detection limit in Europe (*21*). As mentioned earlier, the intakes have been estimated at less than 0.00003 mg/kg bw/day for a 60-kg person for each of these compounds.

Chlordane was evaluated by JMPR in 1986 when an ADI of 0 to 0.0005 mg/kg bw was established (*25*). An RfD for noncarcinogenic effects of chlordane has been established by the U.S. EPA at 0.00006 mg/kg bw/day based on a no effect level of 0.055 mg/kg bw/day in a rat chronic bio-assays. At 0.273 mg/kg bw/day, regional liver hypertrophy was observed in females. The safety factor was 1000 (*5*).

In a 24-month chronic toxicity study in the mouse, a NOEL of 0.15 mg/kg bw/day was observed based on hepatocellular swelling and necrosis in males and increased liver weight in males and females at 0.75 mg/kg bw/day (*5*). Chlordane has been studied in four mouse and four rat long-term carcinogenesis bioassays. Dose-related incidences of liver carcinoma constitute the major finding in mice. In the rat, chlordane produced a significant increase in adenomas of the liver of male rats. Although no tumors were observed in female rats, hepatocellular swelling was significantly increased. Other studies in the rat have been negative (*5*).

Most studies have found chlordane not to be genotoxic. No adequate studies on reproduction and teratology are available (*5*).

Heptachlor was evaluated by JMPR in 1991 when an ADI of 0 to 0.0001 mg/kg bw was established for heptachlor and heptachlor epoxide combined (*23*). An RfD for noncarcinogenic effects was established by U.S. EPA at 0.0005 mg/kg bw/day based on a no effect level of 0.15 mg/kg bw/day in a 2-year chronic feeding study in rats. At 0.25 mg/kg bw/day, increased liver weight was seen in males. The safety factor was 300 (*5*).

In an inadequate three-generation reproduction study, no effects were observed after 0.5 mg/kg bw/day (*5*).

Heptachlor induced hepatocellular carcinomas in male and female mice in two studies. No indication of treatment-related increase of tumors has been reported in chronic studies with rats.

Most studies have indicated that heptachlor is not mutagenic.

Heptachlor epoxide was evaluated by JMPR in 1991 when an ADI of 0 to 0.0001 mg/kg bw was established for heptachlor and heptachlor epoxide combined (*23*). An RfD for noncarcinogenic effects was established by the U.S. EPA at 0.000013 mg/kg bw/day based on a lowest effect level of 0.0125 mg/kg bw/day in a 60-week chronic feeding study in dogs. The effect seen was increased liver-to-body weight in both males and females. The safety factor was 1000 (*5*).

A NOEL of 0.025 mg/kg bw/day was established from a two-generation reproduction study in dogs. At 0.075 mg/kg bw/day, liver lesions were observed in pups. Reduced pup survival was seen after 0.175 mg/kg bw/day. In a three-generation reproduction study in rats the NOEL was 0.25 mg/kg bw/day. At 0.5 mg/kg bw/day, increased pup mortality was recorded (*5*).

Heptachlor epoxide has induced liver carcinomas in two strains of mice of both sexes. In rats no significant carcinogenic effect was seen.

Most studies have indicated that heptachlor epoxide is not mutagenic.

Dieldrin. Dieldrin is the major metabolite of aldrin. Both compounds have been severely restricted or banned since the early 1970s in a number of countries. Dieldrin accumulates in the mammalian organism. Aldrin is rarely found in food. Dieldrin levels in food (total diet study, United Kingdom) have been declining from 0.004 mg/kg in 1966 to 1967 to 0.0005 mg/kg in 1981 (*46*). In 1986, dieldrin levels in human milk in Denmark ranged from less than 0.01 to 0.04 mg/kg fat (*21*). The intake has been estimated at less than 0.00003 mg/kg bw/day for a 60-kg person.

JMPR in 1977 established an ADI at 0 to 0.0001 mg/kg bw/day for the combined total of dieldrin and aldrin (*22*).

An RfD for noncarcinogenic effects was established by the U.S. EPA at 0.00005 mg/kg bw/day based on a no effect level of 0.005 mg/kg bw/day in a 2-year feeding study in rats. At 0.05 mg/kg bw/day, increased liver weight and liver lesions (parenchymal cell changes including focal proliferation and focal hyperplasia) were observed. The safety factor was 100 (*5*). Liver toxicity of dieldrin has also been observed in the mouse, hamster, dog, and monkey in chronic feeding studies (*46*).

In most of the reproduction studies (over one to six generations) carried out with aldrin or dieldrin on mice and rats, the major effect was an increased mortality rate in pups not yet weaned. Reproductive performance was only affected at doses causing maternal intoxication. It was concluded, that 0.1 mg/kg bw/day was a NOAEL in the rat (*46*).

Dieldrin was carcinogenic in seven strains of mice when administered orally. Dieldrin increased the incidence of hepatomas and hepatic carcinomas. It also produced significant increases in the incidence of pulmonary adenomas, pulmonary carcinomas, and lymphoid tumors. However, in the rat, seven studies with four strains of rats fed 0.1 to 285 ppm dieldrin and varying in duration of exposure from 80 weeks to 31 months did not produce positive results for carcinogenicity (*5*).

Although positive in a few assays *in vitro* for clastogenic activity, most studies *in vitro* and *in vivo* on the genotoxicity of aldrin and dieldrin have yielded negative results (46).

Mirex. Mirex was commonly used in the United States in the 1960s and 1970s to control fire ants; it was also used in plastics, rubber, paints, paper, and electrical goods, but its use is now banned. The intake has been estimated at less than 0.00003 mg/kg bw/day for a 60-kg person.

An RfD for noncarcinogenic effects was established by the U.S. EPA at 0.0002 mg/kg bw/day based on a no effect level of 0.07 mg/kg bw/day in a chronic feeding study in rats. The effects seen at at a dose level of 0.7 mg/kg bw/day were liver hypertrophy and thyroid cystic follicles. Histologic examinations revealed dose-related changes in the parathyroid gland, kidney, liver, spleen, and thyroid. The safety factor was 300 (*5*). Effects of mirex on the liver and thyroid have been reported in many other studies.

Effects on the testis have been reported. Histologic evaluation of the testis revealed hypocellularity, decreased spermatogenesis, and luminal nucleated and giant cells, all of which are characteristic of testicular degeneration. Reproductive and developmental effects (decreased fertility, fetal cataracts, edema) have been reported in several studies. Perinatal and postnatal exposure to mirex resulted in significantly decreased pup survival (*5*).

Mirex is an inducer of liver microsomal mixed function oxidases.

Toxaphene (Camphechlor). Toxaphene is a mixture of several hundred polychlorinated terpenes. Toxaphene is a global pollutant spread by long-range air transportation. Although hardly used in Sweden, it was detected in two pooled human milk samples; the level calculated from 22 peaks was 0.1 ppm in the milk fat.

The intake has been estimated at less than 0.00003 mg/kg bw/day for a 60-kg person. Toxaphene was evaluated by JMPR in 1973. No ADI was established (*22*). No RfD has been established by U.S. EPA.

Dietary toxaphene administered to mice at doses higher than 50 ppm caused dose-related increased incidences of hepatocellular carcinomas and adenomas in both sexes (*5*).

In rats receiving toxaphene at doses of 556 and 1112 ppm for males and 540 and 1080 ppm for females for 80 weeks, a statistically significant dose-related increased incidence of thyroid tumors (adenomas and carcinomas) was seen in both male and female rats.

Toxaphene was mutagenic in bacteria, but negative in a modified dominant lethal assay of male mice (*5*).

Nematocides

Aldicarb. The intake of aldicarb through foods is estimated to be very low and insignificant. Aldicarb does not accumulate in the body.

In 1992 JMPR established an ADI at 0.003 mg/kg bw based on a no effect level of 0.025 mg/kg bw in humans. The no effect level was based on decrease of acetyl cholinesterase (Ache) activity in erythrocytes at 0.05 mg/kg bw (*35*).

An RfD for noncarcinogenic effects was established by the U.S. EPA at 0.001 mg/kg bw/day based on a no effect level of 0.01 mg/kg bw/day in acute oral exposure studies in humans. Essentially all hazards from aldicarb exposure are associated with cholinesterase inhibition. At 0.025 mg/kg bw/day, clinical signs of acetylcholinesterase inhibition were observed. The safety factor was 10 (*5*).

Available evidence both from experimental animals and from humans exposed to aldicarb suggests that neurobehavioral effects are short lasting with no accumulation of effects over time. Thus, the doses producing effects following repeated daily exposure are comparable to those following a single dose. Also, a comparable degree of cholinesterase inhibition is seen at the same dose levels, whether delivered in one acute dose or by subchronic or chronic dosing (*5*).

The results of a 2-year feeding study in the rat demonstrated that the exposed animals did not differ significantly from controls for any of a number of toxicological parameters, except the inhibition of cholinesterase. Therefore, the NOAEL for systemic toxicity is greater than or equal to 0.3 mg/kg bw/day (*5*).

In two multigeneration reproduction studies in rats, the lowest NOEL for fetotoxicity was found at 0.3 mg/kg bw/day. No reproductive effects were noted at any dose tested. Decreased body weight of second-generation pups was observed at 0.7 mg/kg bw/day. Reduced pup viability was observed at lactation day 4 at higher doses.

Developmental toxicity studies in rats and rabbits revealed no congenital malformations but showed feto/embryotoxicity at maternally toxic doses. The lowest NOEL for developmental effects was 0.125 mg/kg bw/day in the rat and 0.25 mg/kg bw/day in the rabbit.

Aldicarb was not found to induce significant increases in tumor incidence in mice or rats in feeding studies or mice in a skin-painting study. In the feeding studies there were, however, significant changes in the incidence of pituitary tumors in female rats and fibrosarcomas in the male mouse (*5*).

Aldicarb has not been shown to be genotoxic in any of a variety of *in vitro* and *in vivo* genotoxicity assays (*35*).

1,2-Dibromo-3-chloropropane. No information on exposure to 1,2-dibromo-3-chloropropane (DBCP) is available.

DBCP has not been evaluated by JMPR and an oral RfD is not available from the U.S. EPA.

An inhalation RfD for noncarcinogenic effects was established by the U.S. EPA at 0.0002 mg/m^3 based on a no effect level of 0.17 mg/m^3 in a 13-week, subchronic rabbit inhalation study. At 1.7 mg/m^3 testicular effects were observed. The safety factor was 1000 (*5*). In that study, the average sperm count as well as the percentage of live sperm of the rabbits was significantly less than those of the controls after 7 weeks of exposure, and remained decreased for the duration of the exposure period and through week 42 after the exposure. To assess the effects of DBCP on fertility, exposed male rabbits were mated to unexposed female rabbits at weeks 14 and 41 of the study. DBCP did not affect the libido of the exposed male rabbits during week 14; however, the five males exposed to 17 mg/m^3 DBCP were infertile. During week 41 (27 weeks postexposure), all rabbits exposed to lower concentrations of DBCP produced normal litters, and two of the five males exposed to 17 mg/m^3 regained fertility (i.e., increased sperm count) and produced normal litters. The FSH serum levels were also significantly elevated at 14 weeks in the males exposed to 1.7 mg/m^3 DBCP. Increased FSH serum levels were consistent with a marked decrease in sperm count, whereas serum levels of testosterone were unchanged. The only gross lesion observed under macroscopic examination was the small size of the testes. A histopathologic examination revealed changes in the reproductive system. These effects included atrophy of the testes, epididymides, and accessory sex glands including the prostate. The testes weight was significantly decreased to 50% of control values (week 14) in the group exposed to 1.7 mg/m^3 and to 75% of control values (week 8) in the group exposed to 17 mg/m^3. Severe testicular atrophy was characterized by nearly complete or complete loss of spermatogenic cells in seminiferous

tubules. Following the recovery period, tubular regeneration was observed in the testes of the affected rabbits (*5*).

A number of occupational studies on exposure to DBCP demonstrated this compound to be a potent testicular toxicant in humans. The azoospermic men had elevated FSH, but normal LH and testosterone levels. Testicular biopsy showed atrophy of seminiferous epithelium and tubules lined by Sertoli cells only. Few reliable exposure data are available from any of these studies. Limited follow-up studies of affected worker populations indicate that paternal exposure to DBCP sufficient to produce oligoospermia or azoospermia did not detectably increase the rate of congenital malformations or impair the health status of offspring conceived during or after DBCP exposure (*5*).

DBCP is, however, a potential mutagen capable of inducing a dominant-lethal effect in mice.

Chronic inhalation carcinogenesis bioassays in rats and mice have established DBCP as a carcinogen, with high incidences of tumors appearing in the nasal cavity and on the tongues of rats and in the nasal cavity and lungs of mice (*5*).

Industrial Chemicals and Environmental Pollutants

Bisphenol-A. Bisphenol-A is used as a plastic monomer in the manufacture of epoxy, polycarbonate, and polyester–styrene resins. Little is known about exposure to bisphenol-A. Recently, bisphenol-A was found to be released from the epoxy–lacquer coating in food cans. Concentrations from 0.004 to 0.023 mg per kg of food have been detected in canned food (*47*).

A tolerable daily intake of bisphenol-A was established at 0.05 mg/kg bw by the EU Scientific Committee for Food and a specific limit for migration of bisphenol-A from food packaging materials has been set at 3 mg per kg of food (*48*).

An RfD for chronic oral exposure of bisphenol- A was established at 0.05 mg/kg bw/day by the U.S. EPA (*5*).

In both evaluations an LOAEL of 1000 ppm in the diet (corresponding to 50 mg/kg bw/day) of rats in a 103-week chronic oral bioassay was used applying a safety factor of 1000. Groups of 50 rats of each sex were fed diets containing 0, 1000, or 2000 ppm of bisphenol-A. All treated groups of rats had reduced body weights. Food consumption was also reduced.

In the same study, male mice (50/group) were fed diets containing 0, 1000, or 5000 ppm of bisphenol-A and female mice (50/group) were fed 0, 5000, or 10,000 ppm of bisphenol-A. Male mice at 5000 ppm and female mice at 5000 and 10,000 had reduced body weights. At 1000 and 5000 ppm, there was an increase in the number of multinucleated giant hepatocytes in male mice. This effect was not considered adverse, and an NOAEL in mice was established at 130 mg/kg bw/day (*5*).

The only toxic effect seen in beagle dogs fed 1000 to 9000 ppm of bisphenol-A in the diet for 90 days was an increase in mean liver weight in the high-dose group.

In a two-generation feeding study of bisphenol-A (100–9000 ppm) in rats, decreases in body weight in the parent generation at 9000 ppm and in the F_1 generation at greater than or equal to 1000 ppm were observed. In mice, a dose level of 1250 mg/kg bw/day was associated with fetotoxicity and maternal toxicity, but there was no significant increase in the incidence of malformations at any dose level. In rats, dose levels of less than or equal to 1280 mg/kg/day were not toxic and did not cause malformations to the fetus (*5*).

Polychlorinated Biphenyls. The polychlorinated biphenyls (PCBs) are a series of 209 congeners. They were marketed under various trade names (Aroclor, Clophen A, and Kanechlor). Within each series there were different average chlorine contents in the mixtures. PCBs had diverse uses in electrical transformers and other electrical appliances (*49*), but their use has been severely restricted in most countries since the 1970s and 1980s. Waste containing >50 ppm PCBs should be treated as hazardous waste and destroyed by combustion (*50*). Many of the PCB congeners are very stable in the environment and accumulate in marine, avian, and mammalian species. Foodstuffs, in particular fatty fish, meat, and dairy products, are important sources of human exposure to PCBs. Until recently, most analytical procedures for PCBs in food and human tissue have measured total PCBs and do not reflect differences in composition. Modern analytic techniques are now able to provide congener-specific information, but so far only a limited database has emerged.

The biological effects caused by the various congeners differ, not only in potency but also qualitatively. Several non- and mono-*ortho*-substituted PCB congeners induce effects similar to those caused by chlorinated dioxins and dibenzofurans, i.e., the toxicity is probably mediated through interaction with the Ah receptor, and they are potent inducers of certain cytochrome P450 isozymes (CYP1A1 and CYP1A2). The toxicity of such PCB congeners can be expressed in terms of TEFs, that is, expressed as a fraction of the toxicity of TCDD (*51,52*). Other PCB congeners presumably act by different mechanisms and are potent inducers of a different set of cytochromes (CYP2B1 and CYP2B2). In addition, there are PCB congeners that are intermediate in this respect, i.e., they elicit a mixed spectrum of effects. Our knowledge of possible interactions between the various groups of PCBs is still very limited (*50*).

PCB levels in foods (herring and cod liver) and human milk have declined in the Nordic countries since the 1970s (*2,50*). PCB levels in fish now seem to have stabilized at 10% of the levels found in the beginning of the 1970s. In fact, the levels in herring are now below 0.1 mg/kg. It has been estimated that the intake of PCBs from heavy fish consumption is less than 25 fg per person per day, corresponding to 0.0004 mg/kg bw/day for a 60-kg person.

A Nordic group of experts concluded that the present database does not allow a traditional risk assessment to be performed, i.e., it was not possible to recommend a tolerable daily intake of either total PCB or any individual congener. Furthermore, the evaluation suggested that the present exposure of Nordic populations is of the same order of magnitude as that at which subtle health effects may occur in children exposed *in utero* and possibly through breast-feeding (see "Exposure of Humans to Environmental Chemicals with Estrogenic Activity and Their Effects on Male Reproductive Health"). Further studies would be necessary to clarify whether such effects actually occur (*50*). In addition, when the concentrations of the dioxinlike PCBs are taken into account, the joint risk from PCBs in mothers' milk and certain other foods appears to be of similar importance as the dioxin content.

Most of the animal studies with PCB mixtures have been performed using commercial mixtures that differ in composition from the PCB mixtures to which humans are exposed through food. Although animal feeding studies demonstrate the carcinogenicity, immunotoxicity, and reproductive toxicity of commercial PCB preparations, it is not known which of the PCB congeners in such preparations are responsible for these effects.

PCBs (Aroclor 1260, Kanechlor 500 and Clophen A 60, mixtures containing

more highly chlorinated biphenyls) have produced hepatocellular carcinomas in three strains of rats and two strains of mice. Aroclor 1254 (lower chlorine percentage) only produced hepatocellular adenomas in mice and rats (*5*). Most genotoxicity assays of PCBs have been negative.

PCB exposure has been reported to cause several effects on different processes in reproduction and the development of the embryo/fetus/offspring. Most effects are specific to fertility and to the development of offspring. In addition, teratogenic effects, such as hydronephrosis and cleft palate, have been produced in mice. Effects seen on male reproduction include decreases in fertility, matings and pregnancies; testicular spermatozoa concentration; ventral prostate weight; and seminal vesicle weight. The underlying cause of some of the reproductive effects of PCBs may be alterations in hormonal levels and/or receptor affinities/levels. Decreased levels of gonadal hormones can be explained by enhanced metabolism of steroids, which are the normal substrates for microsomal enzymes induced by PCBs (*50*). In addition, effects on the thyroid gland and its function have frequently been reported (*52*).

Perinatal toxicity and long-term neurobehavioral effects of Aroclor 1016 have been evaluated in infant monkeys. Aroclor 1016 was administered to groups of eight adult female rhesus monkeys via the diet at levels of 0.007 and 0.028 mg/kg bw/day for approximately 22 months. Exposure began 7 months prior to breeding and continued until offspring were weaned at age 4 months. No exposure-related effects were seen in the mothers, who all had uncomplicated pregnancies. Mean birth weights of the infants in the 0.028 mg/kg bw/day dose group were lowered. Effects occurring in the offspring of these monkeys consisted of hairline hyperpigmentation at greater than or equal to 0.007 mg/kg bw/day, and decreased birth weight at 0.028 mg/kg bw/day. The results of neurobehavioral tests in the monkey offspring at 14 months and 4 to 6 years of age demonstrated adverse learning deficits at the 0.028 mg/kg/day maternal dose. However, evaluation of these data is complicated by possible inconsistencies in the outcome of the learning tests. Learning was impaired and facilitated on different problems; performance was improved in the low-dose group, and no significant differences were seen between either test group and the control group. There is some evidence to suggest that deficits in learning are related to decreased brain dopamine, which has been observed in monkeys orally exposed to Aroclor 1016 (*5*). The issue of male reproductive effects was not addressed in these studies.

In the rat, Aroclor 1016 produced elevated serum levels of adrenal cortical hormones, and Aroclor 1016 was immunotoxic in mice.

Aroclor 1016 (3.8 mg/kg bw/day for 237 days) adversely affected reproduction in mink. The investigators noted that the adverse effects on reproduction did not appear to be due to an effect on spermatogenesis, since PCB-treated male mink have had acceptable levels of reproduction when mated to untreated females in other studies (*5*).

In rhesus monkey infants whose mothers were or had been exposed to Aroclor 1248 during gestation and lactation, behavioral testing showed hyperactivity and retarded learning ability at 6 to 24 months of age. However, at 44 months of age the monkeys were hypoactive and at 4 to 6 years of age they showed impairment in learning and memory tests. These effects were reported at doses of about 0.006 mg Aroclor 1248/kg bw/day to the mothers (*50*).

Adult female rhesus monkeys fed 0, 0.1 and 0.2 mg/kg bw/day of Aroclor 1248 for up to 14 months showed skin changes, such as hyperpigmentation and alopecia, characteristic signs of PCB intoxication. Increased menstrual duration was noted. In a breeding trial a highly increased rate of abortion was observed, and liveborn infants showed clinical signs of PCB toxicity and died at weaning. Thymic atrophy was a common lesion in such infants. The study included three recovery breeding periods after dosing: at 22 months, 36 months, and 55 months after the initiation of Aroclor 1248 dosing, respectively. Results of this prolonged recovery period revealed impairment of reproductive function in female rhesus monkeys lasting for more than 4 years after dosing ceased. In the groups of infants for which birth weight data are available, a significant reduction in mean birth weight for PCB-exposed infants was evident. Severe immunological deficiencies have also been observed in monkeys after PCB exposure.

Studies of individual congeners and PCB mixtures of higher chlorine content in animals indicate, in general, that PCBs are readily and extensively absorbed (75–>90%) in rats, mice, monkeys, and ferrets. A study of a PCB mixture containing 54% chlorine provides direct evidence of absorption of PCBs in humans after oral exposure, and indirect evidence of oral absorption of PCBs by humans is available from studies of ingestion of contaminated fish by the general population. PCBs distribute preferentially to adipose tissue and concentrate in human breast milk due to its high fat content (*5*).

The estrogenic activity of hydroxylated PCB metabolites has been discussed in the "Environmental Chemicals with Known Estrogenic Effects."

Two episodes of ingestion of PCB-contaminated rice oil have been reported: the Yusho incident of 1968 in Japan and the Yu-Cheng incident of 1979 in Taiwan (see "Exposure of Humans to Environmental Chemicals with Estrogenic Activity and Their Effects on Male Reproductive Health"). There is strong evidence indicating that the health effects seen in Yusho victims were due to ingestion of polychlorinated dibenzofurans, rather than to PCBs themselves (*5*).

Polychlorinated Dibenzo-p-dioxins and Dibenzofurans. Polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans consist of 75 and 135 different congeners, respectively. The most toxic and well-studied congener is TCDD. These compounds are not used commercially but are formed as unwanted byproducts in a variety of chemical and thermal processes, such as production of chlorinated compounds, incineration processes, paper and pulp bleaching, and emissions from steel foundries and from motor vehicles (*53*). These chemical and thermal processes lead to continuous formation and release of PCDDs and PCDFs into the environment.

Although many of the PCDD/PCDFs are emitted into the environment, only congeners with chlorine substitution, at least in positions 2, 3, 7, and 8, are found in biological material. These stable, lipophilic substances accumulate in food chains. The stable PCDD/PCDFs share a common mechanism of action. They have been shown to act via a cytosolic receptor, the Ah receptor. The Ah receptor regulates a number of cellular proteins, such as cytochrome P4501A1 (CYP1A1), and most of the toxic responses following TCDD exposure are believed to be mediated through the Ah receptor. To express the combined toxicity of the PCDD/PCDF mixtures to which humans are exposed, the concept of TCDD equivalencies (TCDD-TEQs) has been developed. In a variety of short-term tests, the toxicity

of each single congener has been expressed as a fraction of the well-known toxicity of TCDD, the so-called TEFs (*54*).

Food is considered to be the main source of exposure to PCDD/PCDFs. The most important food items are milk and milk products, meat and meat products, eggs, and fish and fish products. Data from Germany (1991) have estimated an average daily intake of TCDD-TEQs of 130.3 pg/person/day, corresponding to approximately 2 pg/kg bw/day for a 60-kg person (0.000000002 mg/kg bw/day) (*55*). Comparable results are obtained from a more limited investigation of Danish foodstuffs (*56*). TCDD-TEQs were estimated in samples of mothers milk from Denmark, Sweden, and Norway in 1987. The levels in the three Nordic countries were very similar, the mean values ranged from 17 to 20 pg/g on a fat basis (*54*).

In 1988 a Nordic expert group established a TWI of 35 pg/kg bw/week for TCDD (5 pg/kg bw/day) (*54*). The evaluation was based on a NOAEL of 1000 pg/kg bw/day found in a chronic long-term toxicity/carcinogenicity study in rats and a three-generation reproduction study in rats. The safety factor used was 200. In both studies effects were observed following administration of 10,000 pg/kg bw/day. In the long-term study, liver toxicity and hyperplastic nodules were observed in female rats. At a 10-fold higher dose, female rats developed hepatocellular carcinomas. In the reproduction study 10,000 pg/kg bw/day resulted in decreased litter size, decreased fetal and neonatal survival, and decreased weight gain in surviving pups.

An overwhelming biochemical and toxicological database exists on PCDDs and PCDFs. These compounds produce a wide spectrum of responses in experimental animals and, presumably, in humans. The toxic effects include teratogenic, reproductive, behavioral, neuroendocrine, immunotoxic, and hepatotoxic effects. The tumorigenic actions seen in some rodents most likely are not the result of a genotoxic, but rather the resulst of a promotional, and possibly hormonal, mechanism (*53*). The biochemical effects include induction of a number of cytochromes, most noticeably CYP1A1 and CYP1A2. Indirect effects, such as alterations of hormonal metabolism leading to, for example, altered sex hormone levels and lowered thyroid hormone levels may have an influence on growth and development of the organism. Most of the toxic and biological responses are thought to be initiated through the binding to the Ah receptor. This binding is followed by translocation to the dioxin-responsive enhancer (DRE) elements on DNA for the expression of many of the species- and tissue-specific, toxicological and biological responses, including increased expression of growth factors. However, there is some evidence that some effects, e.g., alteration in thyroid hormone metabolism, are not necessarily Ah-receptor-mediated.

The effect of TCDD on male reproduction in the rat has already been discussed in "Environmental Chemicals with Known Estrogenic Effects." TCDD is both antiestrogenic and antiandrogenic. A comprehensive review of a number of endocrine and other effects of TCDD has been given by Pohjanvirta and Tuomisto (*57*).

Pentachlorophenol. Pentachlorophenol (PCP) has been widely used as a wood preservative. Dietary exposure to PCP is thought to be limited. An average intake of 0.00015 mg/kg bw/day has been estimated. PCP may be present in indoor and outdoor air, but data on inhalation exposure are scarce (*58*).

A RfD for noncarcinogenic effects was established by the U.S. EPA at 0.03 mg/kg bw/day based on a no effect level of 3 mg/kg bw/day in a 2-year chronic feeding study in rats. At 10 mg/kg bw/day pigmentation of liver and kidneys was observed in females. The safety factor used was 100 (*5*). The main organs or systems affected in animals after long-term exposure to low levels of PCP were the liver, kidney, nervous system, and immune system (*58*).

Numerous studies have investigated the teratogenicity of PCP administered orally to rodents. These studies did not reveal teratogenic effects; however, feto/maternal toxicity was seen at 30 mg/kg bw/day. Since PCP apparently does not cross the placental barrier, the observed fetotoxicity may be a reflection of maternal toxicity (*5*).

In mice, PCP exposure increased the incidences of hepatocellular adenomas and carcinomas, adrenal medulla pheochromocytomas and malignant pheochromocytomas, and/or hemangiosarcomas and hemangiomas in one or both sexes. In two chronic oral rat studies no significant increase in tumor incidence as compared with controls was observed.

Mutagenicity data provides some indication that PCP has clastogenic potential (*5*).

Alkylphenol Ethoxylates. Alkylphenol ethoxylates (APEs) are nonionic surfactants widely used as detergents, emulsifiers, wetting agents, and dispersing agents in household products and in agricultural and industrial applications. They are also used as spermicides in contraceptive foams, jellies, and creams. The alkyl group typically is a branched nonyl-, octyl- or dodecyl-chain. The ethoxylate chain may have 1 to 100 repeating units. Nonylphenol ethoxylates (NPEs; nonoxynols) are the most common forms of the APEs.

Alkylphenol, alkylphenol mono- and diethoxylates, and their carboxylic acids are the products of the microbial breakdown of APEs and are present in surface waters and sediments. The alkylphenols are relatively persistent and bioaccumulate in the lipid of living organisms (see section "Environmental Chemicals with Known Estrogenic Effects"). In addition to the obvious exposure from the use of spermicides and contact with products containing APEs, it is possible that humans can be exposed to APE degradation products through the water supply, sewage sludge used for fertilizer, and aquatic organisms used as food. However, virtually nothing is known about the magnitude of human exposure to these compounds.

Nonoxynol-9 (9 ethylene oxide units), which is used as a spermicide, was rapidly absorbed from the vagina of rats (*59*). There is also evidence that nonoxynol-9 can be metabolized to nonylphenol, which after glucuronidation was excreted through urine and bile (*60*). Intravaginal administration of nonoxynol-9 in rats caused damage to the vaginal epithelium and acute inflammation in the vagina, cervix, and uterus (*61*). When administered to rats during the first week of pregnancy, nonoxynol was found to be embryotoxic (*62*).

Meyer et al. (*63*) observed fetotoxicity of nonoxynol-9 at a high oral dose level (500 mg/kg bw/day) that also caused maternal toxicity. When given epicutaneously, no effect was seen. Nonoxynol-30 was without effects. Nonoxynol-9 was not mutagenic in the Ames test (*63*).

Octylphenol and nonylphenol were found to be estrogenic both *in vitro* and *in vivo* (see "Environmental Chemicals with Known Estrogenic Effects").

Phthalate Esters. Since the 1920s phthalate esters have been used as plasticizers for polyvinyl chloride. The most widely used phthalate ester is di(2-ethylhexyl)-phthalate (DEHP) (comprising 50% of all phthalate ester plasticizers). It may account for 40% or more of the plastic. In addition to this ester, a range of phthalate esters is available and used in different plastics, depending on the intended use of the

product. Examples are dibutyl phthalate (DBP) and butylbenzyl phthalate (BBP). Phthalate esters are produced in enormous quantities. They are present in many commercially available plastics and are leachable or volatilizable from such plastics. The environmental fate of phthalate esters after their release from plastics has not been widely studied (*64*).

DEHP exists widely in the environment and is found in most samples, including air, precipitation, water, sediment, soil, and biota. Levels up to 300 ng/m^3 have been found in urban air. River sediment levels up to 70 mg/kg have been reported. DEHP also has been found in various types of food, such as fish, shellfish, eggs, and cheese. The average dietary exposure to DEHP was estimated at 300 fg/person/day in the United States in 1974. Blood transfusions and other medical treatments using plastic devices may lead to involuntary human exposure to DEHP (*65*). When all sources were considered, the total dietary intake of DEHP in the United States was estimated to be 5.8 mg/person/day. Maximum exposure to total phthalate from packaging sources alone in the United Kingdom was estimated at 4.37 mg/person/day [cited by Sharman et al. (*66*)]. Sharman et al. (*66*) found total phthalates in dairy products (in the United Kingdom) ranging from 4 to 20 mg/kg in cheese and butter (DEHP from 0.6–3 mg/kg).

The extent of human exposure to phthalate esters in Denmark is not known. In Denmark, DEHP is not allowed for use in plastic materials intended to come into contact with foods. Petersen (*67*) found a mean concentration of DEHP lower than 0.05 mg/liter in retail whole milk from Denmark.

The European Union Scientific Committee for Food has established an ADI of 0.025 mg/kg bw/day for DEHP (*67*). An RfD for non-carcinogenic effects of DEHP was established by the U.S. EPA at 0.02 mg/kg bw/day based on an LOAEL of 19 mg/kg bw/day in a subchronic to chronic feeding study in guinea pigs. The effect observed was increased liver weight. The safety factor was 1000 (*5*).

MEHP, the monoester form of DEHP is the principal metabolite of DEHP (*65*). Several studies have shown testicular atrophy after DEHP administration (high doses). Younger rats seem to be more susceptible than older ones, and rats and mice may be more sensitive than hamsters and monkeys. The effect was reversible. MEHP has toxic effects on Sertoli cells *in vitro* (*65*).

Dietary levels of 0, 0.01, 0.1, and 0.3% DEHP were administered to male and female CD-1 mice that were examined for adverse fertility and reproductive effects using a continuous-breeding protocol. DEHP was a reproductive toxicant in both sexes, significantly decreasing fertility and the proportion of pups born alive per litter at the 0.3% level, and inducing damage to the seminiferous tubules. DEHP has been observed to be both fetotoxic and teratogenic.

DEHP is a potent inducer of hepatic peroxisomal enzyme activity. This effect is strongly associated with the hepatocarcinogenic effect of DEHP. In contrast to rat hepatocytes, DEHP metabolites do not produce peroxisome proliferation in human hepatocytes (*65*). In a long-term carcinogenicity study, groups of rats were fed with 0, 300, or 600 mg DEHP/kg bw/day for 103 weeks. Similarly, groups of mice received DEHP in the diet for 103 weeks. No clinical signs of toxicity were observed in either rats or mice. A statistically significant increase in the incidence of hepatocellular carcinomas and the combined incidence of carcinomas and adenomas was observed in female rats and both sexes of mice.

Studies indicate that DEHP is not a directly acting mutagen. MEHP, the monoester form of DEHP and a metabolite, was positive in several assays for mutagenicity and clastogenicity (*65*).

An RfD for noncarcinogenic effects of DBP was established by the U.S. EPA at 0.1 mg/kg bw/day based on an NOAEL of 125 mg/kg bw/day in a subchronic to chronic feeding study in rats. The effect observed after 600 mg/kg bw/day was increased mortality. The safety factor was 1000 (*5*). Fetotoxicity was observed when mice were fed 2100 mg/kg bw/day DBP throughout gestation. An increase in terata of borderline statistical significance was observed in progeny of this treatment group.

DBP causes degeneration of the seminiferous tubules, probably as a result of increased urinary excretion of zinc. No data on carcinogenicity are available.

DBP did not induce mutations in bacteria. It was mutagenic in the mouse lymphoma forward mutation assay only in the presence of metabolic activation. In addition, DBP showed some evidence of clastogenic activity in mammalian cells. It is hydrolyzed to monoesters. There is also evidence that DBP induces peroxisome proliferation (5).

An RfD for noncarcinogenic effects of butylbenzyl phthalate (BBP) was established by the U.S. EPA at 0.2 mg/kg bw/day based on an NOAEL of 159 mg/kg bw/day in a 6-month feeding study in rats. The effect observed after 470 mg/kg bw/day was increased liver weight. The safety factor was 1000 (*5*). In the previously mentioned study all rats given 1417 mg/kg bw/day BBP had small testes; 5/11 had soft testes; and 1/11 had a small prostate and seminal vesicle. In addition, testicular lesions characterized by atrophy of seminiferous tubules and aspermia were also observed. At the lower dose levels there were no observable effects on male reproductive organs. A male mating trial study was performed concomitantly with the toxicity study. Testicular atrophy was observed in male rats after 10 weeks of exposure to 2875 mg/kg bw/day). In an older rat study no effects were reported after 250 mg/kg bw/day, while liver weights were increased in animals fed diets containing 500, 750, or 1000 mg/kg bw/day, respectively, for 90 days. A mild decrease in growth rate was reported for the two top dose groups.

In a 14-day rat study, BBP at a dose level of 375 mg/kg bw/day produced significant increases in liver and kidney weights and kidney pathology (proximal tubular regeneration).

In male rats administered 160, 480, or 1600 mg/kg bw/day BBP for 14 days, biochemical or morphological changes in the liver as well as effects on testes weights were not observed in the 160 mg/kg/day dose group. However, at 480 mg/kg bw/day, liver enzyme activities were increased and testicular atrophy was observed in some of the rats. BBP was tested at dietary levels of 0, 6000, and 12,000 ppm in long-term carcinogenicity studies in mice and rats.

BBP induced a statistically significant increase in mononuclear cell leukemia in female rats; the response in male rats was inconclusive and there was no such response in mice. BBP did not induce lung adenomas in strain A mice administered 24 ip injections of 160, 400, or 800 mg/kg bw. BBP is not mutagenic in *in vitro* assays (*5*).

Appendix B: Identification and Assessment of the Effects of Chemicals on Reproduction and Development, Focusing on the Effects on Males

The following effects have to be considered:

- Impairment of male (and female) reproductive functions or capacity, i.e., adverse effects on libido, sexual behavior, any aspect of spermatogenesis (or oogenesis), or hormonal activity or physiological response that would interfere with the capacity to fertilize, fertilization alone, or development of the fertilized ovum up to and including implantation.
- Induction of noninheritable harmful effects on the progeny, i.e., in the broadest sense any effect interfering with normal development, both before and after birth up to puberty, should be included. Both morphological malformation(s) and functional disturbances (e.g., hormonal, neurological) should be evaluated.

The risk assessment includes:

- Hazard identification
- Dose–effect assessment (estimation of a possible NOAEL)
- Extrapolation (prediction of adverse effects in other species, particularly in man)
- Prediction of safe levels of exposure in man

Experimental Studies in Laboratory Animals

Many different experimental methods are being used for the investigation of the reproductive toxicity of chemicals. Several tests are standardized and guidelines have been issued by various governmental agencies and international organizations. Other tests are still undergoing scientific evaluation.

The group of tests discussed below is not a comprehensive listing of all available tests but rather a presentation of representative examples. (The presentation of the individual tests and the test strategies are based on the references given at the end of this appendix)

Tests for Reproductive Toxicity

The description of the tests is based on the following references:

- OECD (*68*)
- Commission of the European Communities (*69*)
- Ministry of Health, The National Food Agency of Denmark (*70*)
- Nordic Council of Ministers and National Institute of Occupational Health, Denmark (*71*)
- Hansen and Meyer (*72*)
- European Chemical Industry, Ecology and Toxicology Centre (ECETOC) (*73*)

One-, Two-, and Multigeneration Studies. Generation studies examine successive generations to identify possible effects of a substance on fertility of male and female animals; pre-, peri-, and postnatal effects on the ovum, fetus, and progeny, including teratogenic and mutagenic effects; and peri- and postnatal effects on the mother.

Various international organizations and countries have drafted guidelines for these tests, with a number of common requirements. The preferred species are the rat and the mouse; other species may be used if relevant (e.g., differences in the toxicokinetics between the preferred species and man to clarify ambiguous results or to further study observed effects). The test substances are administered by the appropriate route to groups of animals (number of animals per group should be sufficient to yield about 20 pregnant animals at or near term). The chemical is administered over at least one spermatogenic cycle and the last stages of oocyte maturation before the parent generation animals are mated. The exposure of the females is continued throughout the mating period and the gestation up to weaning of the last generation. At least three treatment groups and a control group (untreated or vehicle in the highest dose used) should be used. Ideally, unless limited by the physicochemical nature or biological effects of the test substance, the highest dose level should induce toxicity but not mortality in the parental animals. The low dose should ideally not induce any observable adverse effects on the parents or offspring.

The animals are observed daily for clinical changes. Body weight is recorded weekly for the parent animals and for offspring normally at birth, on days 4, 7, and 14, at weaning, and thereafter every week. Pregnancy rate, duration of pregnancy, number of pups per litter, number of live and dead pups, number of pups with anomalies are recorded, and, if necessary, histological examinations of dead or sacrificed animals are performed. The number of live pups on day 4 and at weaning are recorded and the following indices are often calculated:

- Mating index: copulation/estrous cycles required
- Fecundity index: pregnancies/copulations
- Male fertility index: males impregnating females/males exposed to fertile nonpregnant females
- Female fertility index: females conceiving/females exposed to fertile males
- Incidence of parturition: parturitions/pregnancies
- Live birth index: viable pups born/pups born
- Survival index for 24 hr, 4 days, 12 days, and 21 days

The number of litters per female per generation varies in the different guidelines from one to two.

Fertility assessment by continuous breeding has been used to study the depletion of oocytes from the ovary in mice exposed to procarbazine. In this study, prenatally treated mice were housed continuously with untreated male mice, and the cumulative number of offspring was measured by removing the female when she was noticeably pregnant and then returning the female to the male's cage immediately after the birth of her pup in order to establish a pattern of forced repetitive breedings.

In proposed guidelines from the U.S. EPA (*74*), samples of sperm from the distal cauda epididymis (or the proximal vas deferens) shall be collected for the evaluation of the percentage of progressively motile sperm and sperm morphology. In addition, the entire cauda epididymis shall be minced in saline to enumerate the total number of sperm.

A number of useful tests of male reproductive toxicity, which are discussed in detail, for example, by Thomas (*75*), are listed below:

- Testis: size *in situ*, weight, consistency, morphometry, spermatid reserves, DNA flow cytometry, biochemical assays, gene expression
- Epididymis: weight and histology, number of sperm in distal half, sperm motility, sperm morphology, biochemical assays, gene expression
- Accessory sex glands: weight and histology, biochemical assays, gene expression
- Semen: total volume, gel-free volume, sperm concentration, sperm motility,

sperm morphology, hamster egg penetration, cervical mucus penetration
- Sperm (special features): video/cinemicrography, membrane structure, metabolism
- Fertility: ratio—exposed/pregnant women; number of embryos per pregnant female; ratio—viable embryos/corpora lutea; number of 2- to 8-cell eggs; number of abnormal eggs
- Endocrine monitoring: testis—testosterone, dihydrotestosterone, estradiol, estrone, thyroid gland—thyroxin, triiodothyronine pituitary—FSH, LH, prolactin, TSH

End points that are used to indicate reproductive dysfunction include (*76*):
- Decreased libido, impotence
- Abnormal sexual behavior
- Sperm abnormalities: decreased number or motility, morphology
- Subfecundity: abnormal sex organs and/or pubertal development, infertility
- Early fetal loss
- Late fetal loss (stillbirth)
- Intrapartum death
- Death in the first week
- Decreased birth weight
- Prematurity/postmaturity at birth
- Altered sex ratio
- Multiple births, birth defects
- Infant death
- Childhood morbidity

The Conventional Teratology Study. The teratology study is the *in vivo* method for studying embryo–fetal toxicity as a consequence of exposure during pregnancy (e.g., growth retardation, anatomical variations, teratogenicity, lethality). Various international organizations and countries have drafted guidelines for these tests. These guidelines have a number of common requirements. The preferred species include rodents (e.g., rat, mouse) and nonrodents (e.g., rabbit). Other species may be used if relevant (e.g., differences in the toxicokinetics between the preferred species and man to clarify ambiguous results or to further study observed effects).

Young mature virgin females are artificially inseminated or mated with males. The time of mating is established by observation of mating (e.g., rabbits), identification of a plug (mixture of sperm and cellular material from the vagina of rats and mice), vaginal smear (in rats) or by noting the time of insemination (e.g., for pigs and rabbits). Normally, three dose levels and a control group (untreated or vehicle control; the group size is 20 pregnant animals for rats and mice, and 12 for rabbits) are used to establish a dose–effect relationship. The pregnant female rats are exposed during the period of organogenesis, i.e., between day 6 when implantation occurs and day 15. (The corresponding periods for mice and rabbits are days 6–15 and days 6–18, respectively). This period has been found to be the most sensitive to the induction of structural, anatomical malformations (the corresponding sensitive period for humans is between days 18 and 60 of pregnancy). Days 6 to 15 are the indicated dosing period for pregnant rats. However, this may vary depending on the substance administered or whether the effect on a specific organ is to be studied.

The animals are observed daily for clinical changes. Body weight and food consumption are recorded throughout the gestation. The uterus is removed by cesarean section and the uterus and the fetuses are examined the day before anticipated birth. (The dam is examined macroscopically for any structural abnormalities or pathological changes that may have influenced the pregnancy). If dosing is initiated before or at the time of implantation, the preimplantation loss, i.e., the number of embryos lost prior to implantation, is evaluated.

The total number of implantations, i.e., living embryos, dead embryos, and resorption (embryos that die early and are reassimilated, corresponding to early abortions in humans) are noted. The degree of resorption (i.e., the extent to which the embryo has been resorbed) is recorded to establish the time of death of the embryo during the pregnancy.

The fetuses are sexed, weighed, and examined for gross malformations. Retarded growth and effects on visceral and skeletal development are evaluated, including the degree of ossification of the bones.

The Perinatal and Postnatal Studies. Prenatal exposure to chemicals may lead to a range of functional disturbances in the offspring. For example, lead and methylmercury affect brain development but effects on fertility, the immune system, metabolism of foreign substances, and development as a whole have been observed.

Behavioral teratology identifies changes in behavior due to effects on the central nervous system (CNS) and the peripheral nervous system (PNS). As behavior is affected by the function of other organs such as liver, kidneys, and the endocrine system, toxic effects on these organs in offspring may also be reflected in general changes in behavior. No single test is able to reflect the entire complex and intricate function of behavior. For testing behavior, therefore, a range of parameters—a test battery—is used to identify changes in individual functions.

The most frequently used animal species are the rat and the mouse. The guidelines generally recommend groups of 20 animals with dosing from day 15 of gestation to day 21 postgestation, i.e., spanning fetogenesis and the entire lactation period. (However, the recommended dosing period does not cover all events since the CNS is also susceptible to abnormal development during the period of organogenesis. Consequently, dosing is often started earlier, for example, on day 1 or day 6 of the pregnancy). After birth, the number of progeny is recorded and the litters are adjusted so that each contains the same number of pups. To determine whether the chemical substance tested affects the offspring directly through the mother's milk or indirectly by a change in milk production or as a result of a change in the behavior of the exposed mother, cross-fostering may be employed. Cross-fostering is a method by which litters from exposed mothers are reared by control mothers and vice versa.

Studies to identify abnormal development are conducted on individual animals over short or long periods. In the rat, studies last until weaning (although this period does not cover the entire period of brain development as the brain does not attain an approximate adult stage until the age of 6 weeks, which corresponds to 12 to 15 years of age in humans).

Methodology employed in behavioral teratology is described in several reviews. Behavioral teratology tests may generally be grouped into tests of physical development, simple reflexes, motor function, development of the senses, spontaneous activity, learning and memory, and functions of the neurotransmitter systems.

In Vivo Screening Tests

As the capacity for toxicity testing cannot keep up with the number of chemicals in the modern society, there is an increasing demand for new toxicological tests of shorter duration, using fewer resources. Especially for older chemicals where the patent rights no longer exist, or for chemicals introduced into the market years ago when no or few toxicity data were required, it is essential to develop tools for obtaining data for safety assessment. In recent years such new screening tests for

reproductive and developmental toxicity have been developed. By definition, a screening test is limited in scope compared to a conventional test. Data from a screening test indicating a possible toxic potential of a substance identify the substance as one of high priority for further evaluation.

A short presentation of two examples of screening tests for reproductive and developmental toxicity is given below.

The* in Vivo *Teratology Screening Test. This test was introduced as an alternative method to the above-mentioned teratogenicity tests. The hypothesis underlying this test is that most prenatal effects do not just produce specific defects but also are manifested in the postnatal period as a lack of viability and reduced growth.

Pregnant mice (rats can also be used) are exposed to a test substance from day 8 to day 12 of the pregnancy. A control group is not exposed. One dose level is employed (the minimum toxic dose for the mother animal). The mother animals are weighed during the period of exposure. After birth the litter is weighed on the first and third days. Stillborn young and young that die after birth are dissected and examined for defects. The test focuses on malformations as the end points of concern. Results from a validation study have shown that effects on offspring viability or body weight indicate a potential for teratogenic effects, i.e., malformations. A test guideline for this test has not been established.

Reproduction/Developmental Toxicity Screening Tests. Recently OECD has introduced guidelines for screening tests for reproductive toxicity, i.e., the Reproduction/Developmental Toxicity Screening Test as a part of the Screening Information Data Sets (SIDS) for older chemicals that were produced in large volumes (*68*). The Combined Repeat Dose and Reproduction/Developmental Toxicity Screening Test is a combination of the "Repeated Dose Oral Toxicity—Rodent: 28-Day Study" and a reduced one-generation study, whereas the Preliminary Reproduction Toxicity Screening Test is a reduced one-generation study.

The reduced one-generation test has been validated with, among other chemicals, ethylene glycol monoethyl ether (EGME) and cyclophosphamide (CP). EGME showed both systemic and reproductive/developmental effects similar to those previously reported using standard protocols. The study on CP demonstrated most of the known toxicological properties of CP, including developmental toxicity, but not the (expected) adverse effect on spermatogenesis and fertility (the use of lower dose and shorter premating treatment seem to be possible causes for these negative findings).

On the basis of the experience with the tests, the OECD has prepared proposals for two new guidelines (421 and 422), to be included in the Test Guideline Programme. They are being considered for inclusion as new base set tests among the tests used for new chemicals in EU because of a recognition of the need to obtain initial information on reproduction and developmental toxicity at an early phase of hazard identification.

The purpose of the test is to generate limited information concerning the effects of a chemical on male and female reproductive performance such as gonadal function, mating behavior, conception, development of conceptus, and parturition. The test is not considered as an alternative to, nor a replacement for, the existing generation and teratology studies.

The dosing of the animals is initiated 2 weeks prior to mating and continued until the end of the study on postnatal day 4. The number of animals per group (generally at least three test groups and a control group) is at least 10 animals of each sex (expected to provide at least 8 pregnant females per group). Effects on fertility and birth are registered. Live pups are counted and sexed and litters weighed on days 1 and 4 postpartum. The parameters include, among others, a detailed histological examination on the ovaries, testes, and epididymides of at least the highest dosed and the control animals.

Other Toxicity Tests

In Vivo Tests

Other toxicity tests than those mentioned above can reveal effects that indicate a potential of a chemical to interfere with normal reproduction. Thus, in all the toxicological tests involving repeated dosing, including the carcinogenicity test, the gonads and accessory sex organs are subjected to pathological examination including histopathology.

As an example, a rodent test for genetic toxicology (OECD guideline for testing chemicals), the Rodent Dominant Lethal Test should be mentioned. Male animals are exposed to the test substance and mated to untreated virgin females. The various germ-cell stages can be tested separately using sequential mating intervals. The induction of dominant lethal effects causing embryonic or fetal death is evaluated. Dominant lethals are generally accepted to be the result of chromosomal aberrations (structural and numerical anomalies), but gene mutations and toxic effects cannot be excluded.

In Vitro Tests

During recent years many *in vitro* test systems have been proposed as alternatives to whole animal testing for developmental toxicity. These tests are not able to replace animal testing, but can reduce the number of chemicals to be tested with live animals. *In vitro* techniques may also prove useful in the screening of complex chemical mixtures, e.g., in product development (prescreen) or in the screening of closely related chemicals. In addition, *in vitro* tests can be used as a tool for pinpointing the mechanisms underlying a known embryo-fetotoxic effect, and as such provide information that can improve the interpretation of the results and consequently the extrapolation from laboratory animal experiments to humans. No specific test is mentioned here but a reference is given to a recent review.

Test Strategies

Strategies for the assessment of reproductive toxicity of chemical substances has been proposed (*71,76*). The toxicity testing strategies deal (at present) with evidence for reproductive toxic effects, i.e., hazard assessment, but potency should be considered at a later stage. An example is given in Table B1 (*77*).

Significance of Experimental Data and Their Relevance to Man (Extrapolation)

It has been stated that the ultimate proof that a substance is a human teratogen can come only from information on the consequences of human exposure. This statement is valid regarding toxicological effects in general. However, the experience within the area of developmental toxicology (and carcinogenicity) comparing data from human exposure and data from dosing experimental animals (irrespective of the difficulties comparing two different kinds of data sets), and the biology as expressed by Calabrese (*78*) serve as the basis for the use of laboratory animals in predicting possible consequences for humans. Calabrese says the following:

> Cellular structure and biochemistry are remarkably alike across the entire animal kingdom, starting with the lipoprotein cell

Table B1. Reproductive toxicity testing strategy.

Data	Results	Recommended testing
Human data	Clear evidence of developmental toxicity or impaired fertility	No further testing
	Limited evidence of – developmental toxicity – impaired fertility	Teratology study and/or behavioral teratology study Fertility study
Full toxicological data set in experimental animals	Clear evidence of developmental toxicity or impaired fertility	No further testing
	No indication of reproductive toxicity – but neurotoxicity in adult Some indication of reproductive toxicity	No further testing Behavioral teratology study See below
Acute or repeated dose toxicity studies	Testicular effects	Fertility study (males dosed), sperm-test
	Ovarian effects	Fertility study (females dosed)
	Neurotoxicity in adult animals	Fertility study combined with behavioral teratology study
	High acute toxicity	Generation study or OECD ReproTox screening test
Reproductive toxicity studies	Clear evidence of developmental toxicity or impaired fertility	No further studies
	Developmental toxicity at maternally toxic doses	Studies using specific end points and lower doses
	Equivocal evidence of impaired fertility	Repeat study
	Effects on sperm quality only	Epidemiological study of exposed men
In vitro teratogen study	Teratogen effect	Teratology study
	Teratogen effect plus SAR and kinetics indicate effect	No further studies
Data on kinetics/dynamics	Accumulation in fetus, competitive metabolism, etc.	Studies on developmental toxicity
	Hormone analogue, accumulation in testes	Fertility study (relevant sex dosed)
No toxicological data	No indications	Initial toxicity testing followed by studies for reproductive toxicity

Abbreviations: SAR, structure–activity relationship; OECD, Organisation for Economic Cooperation and Development.

membrane, which affects the absorption of xenobiotics into the cell to metabolic processes like glycolysis, the Krebs' cycle, and numerous other aspects of intermediary metabolism. The similarity among animals on the cellular level is so apparent that it serves as the basis upon which scientists have extrapolated or inferred functions from one species to another.

Thus, concerning findings in experimental studies in laboratory animals, demonstrating developmental toxicity (valid for cancer as well) is indicative of a potential human response.

Concerning the predictive value of *in vitro* results and their relevance for the man, the extrapolation between the response in isolated parts of the organism and that of the intact organism has to be considered.

Concerning the predictive value of effects on fertility that were demonstrated in rodent studies and their relevance for the man, the experience is limited. However, it has been stated that effects on fertility in rodents seem to be a good indicator for effects in humans, and most work on contraceptive agents in humans stems from original studies in rodents. In particular, agents causing toxic damage to the testis in animals seem to have a similar effect in humans (*79*).

Present Situation

Chemicals that Have Been Subjected to a Full-scale Toxicological Testing Program

The toxicology testing program includes testing of the chemical, covering exposure in all periods of the lifespan, including the reproductive cycle. Thus, in principle, it should be possible to detect any consequence of an exposure to chemicals at any period, if the test program and the specific tests are correctly designed. However, several questions remain:

- Is the testing program sufficient to identify the possible consequences of exposure?
- Are the effectiveness/sensitivity of the specific toxicological methods sufficient (e.g., number of animals involved per number of species tested per number of groups per dosage regimen). Do we need new or additional toxicological tests?
- Are the parameters used relevant and sufficient? Will the specific test benefit from the introduction of additional parameter(s) or do we need additional parameters (end points)?

Other Chemicals Subjected to Only a Limited Number of Toxicological Tests

New chemicals are subjected to a testing program in the European Union involving different levels of testing according to the tonnage on the market. Most chemicals have only been tested in a limited range of tests according to the requirements on the base set level. Only a few new chemicals will reach a tonnage level triggering the demand for a full scale testing program (level 2).

The existing (older) chemicals are tested according to a strategy that considers the actual use of the chemical/actual exposure and the potential of health hazard. Thus, a few of these chemicals have been subjected to some or more toxicological tests, while only a very limited set of data is available for many of the chemicals. Therefore, the following problems should be discussed:

- What is the possibility of introducing one or more screening tests to obtain data sufficient for an acceptable assessment of the potential effect on reproduction?
- What are the possibilities of introducting additional parameters in the existing tests at the base level to obtain additional information on the potential of the specific chemical to impair the reproduction?

Introduction of additional parameters or even new test methodology within the area of reproductive toxicology will require more experimental work to help us better understand the underlying biological mechanisms.

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